Universidade de Lisboa

Faculdade de Ciências

Departamento de Química e Bioquímica

PHENOLIC COMPOUNDS AND DITERPENOIDS WITH ACTIVITY IN AQUEOUS EXTRACTS OF *PLECTRANTHUS*

Emma Louise Kilner Gomes

Dissertação

MESTRADO EM QUÍMICA

Química

2014

Universidade de Lisboa

Faculdade de Ciências

Departamento de Química e Bioquímica

PHENOLIC COMPOUNDS AND DITERPENOIDS WITH ACTIVITY IN AQUEOUS EXTRACTS OF *PLECTRANTHUS*

Emma Louise Kilner Gomes

Dissertação orientada por:

Professor Doutor Carlos Manuel Ferreira de Sousa Borges

Doutor Paulo Jorge Amorim Madeira

MESTRADO EM QUÍMICA

Química

2014

The work presented in this dissertation was undertaken in the Environmental and Biological Mass Spectrometry Group, Departamento de Química e Bioquímica, Faculdade de Ciências da Universidade de Lisboa, under the guidance of Professor Doutor Carlos Borges e Doutor Paulo Madeira.

Acknowledgements

This work was achieved due to the support of a bunch of people that have been by my side not only during its performance, but also throughout my life.

First, I would like to thank Professor Doutor Carlos Borges and Doutor Paulo Madeira for the guidance, help, motivation and all the knowledge transmitted during these months.

Within the Environmental and Biological Mass Spectrometry Group, I would like to thank first Professora Maria Helena Florêncio for receiving me in her group and for the support during this work. Thanks also to Sofia Alves, Ana Marques, Samuel Barata, Joana Henriques and Daniela Guerreiro for their friendship, support and all the fun and relaxing moments. I also thank Professora Maria Luísa Serralheiro for proving me the plant extracts and for all her help during the work, Professora Doutora Lia Ascensão for the collection and identification of the plants, and Doutor Pedro Falé for the preparation of the extracts and the establishment of the initial LC-MS conditions.

From the Separation Science and Technology Group, Samir Ahmad for his help during the SPE process. From the Organometallic Group, I thank Tânia Morais for her friendship, for all the help and advice during these months, and for all the fun days passed together.

A very special thank you to Tiago Jorge for helping me out when I arrived at the lab, for teaching me how to use all the equipment, for his friendship, jokes and fun moments, and for always being there for my doubts.

Also a special thank you to my friends Filipa, Maria, Raquel and Tiago for always being by my side and supporting me through this stage of my life.

Last but certainly not least, my family, especially my mum and my sister, for always believing in me and supporting my decisions, for all the strength, motivation and friendship during this work and all my life.

Thank you all,

Emma :D

Resumo

Nos dias de hoje, a análise química de alimentos e de plantas tem-se tornado importante não só para cientistas durante a sua investigação, mas tem também influência na rotina diária dos seres humanos.

Este trabalho teve como objetivo principal a identificação de compostos fenólicos e diterpenóides em extratos aquosos de *Plectranthus*. Estas plantas estão em uso há um grande número de anos devido às suas conhecidas propriedades medicinais.

Para começar, realizou-se um estudo do comportamento em estado gasoso para cinco flavonas (acacetina, chrysoeriol, luteolina, orientina e vitexina). Foram efetuados cálculos semi-empíricos para determinar os locais de protonação e desprotonação energeticamente mais favoráveis para as cinco flavonas. Enquanto a protonação é mais provável de ocorrer no grupo C=O da posição 4 para as cinco flavonas, o local de desprotonação mais favorável difere. Estes compostos foram depois analisados por espectrometria de massa tandem com ionização por *electrospray*, os seus iões produto foram estudados e algumas vias de fragmentação foram propostas. Foram observadas várias perdas neutras, juntamente com quebras de ligações C-C no anel C da aglícona e no açúcar, quando aplicável.

Os extratos aquosos de *Plectranthus* previamente preparados foram analisados por cromatografia líquida acoplada à espectrometria de massa. Foram testadas várias condições cromatográficas de modo a obter a melhor separação possível entre os compostos presentes nas amostras. Vários compostos foram identificados nos dez extratos, sendo que o ácido rosmarínico (conhecido pelas suas boas propriedades antioxidantes) foi o único encontrado em todas as amostras e o presente em maior abundância. A percentagem de ácido rosmarínico presente nas amostras foi depois comparada com o potencial antioxidante de cada extrato.

Palavras-chave: *Plectranthus*, compostos fenólicos, diterpenóides, espectrometria de massa, cálculos semi-empíricos.

Abstract

Nowadays, chemical analysis of foodstuffs and plants has become important not only for scientists during their research, but it also has influence in human beings' daily routine.

The main goal of this work consisted in the identification of phenolic compounds and diterpenoids in aqueous extracts of *Plectranthus*. These plants have been in use for a large number of years due to their known medicinal properties.

First, a gas-phase behaviour study was performed for five flavones (acacetin, chrysoeriol, luteolin, orientin and vitexin). Semi-empirical calculations were used to determine the most probable protonation and deprotonation sites for the flavones. While the protonation is predicted to occur at position 4 C=O group for the five flavones, the predicted deprotonation site varies. These compounds were then analysed by electrospray tandem mass spectrometry in the positive and negative ion modes, their product ions were studied and some fragmentation pathways were established. Various neutral losses were observed, together with cross-ring cleavages of the aglycone and the sugar moiety, when applicable.

The previously prepared *Plectranthus* extracts were analysed by liquid chromatography coupled to electrospray mass spectrometry. Several chromatographic conditions were tested to obtain the best separation of the compounds present in the complex samples. Many compounds were identified in the ten extracts, being rosmarinic acid (which possesses great antioxidant properties) the only one present in them all and the most abundant one. The percentage of rosmarinic acid found in the samples was then compared with the antioxidant potential of each extract.

Keywords: *Plectranthus*, phenolic compounds, diterpenoids, mass spectrometry, semi-empirical calculations.

Resumo da dissertação de mestrado em português

"Compostos fenólicos e diterpenóides com atividade em extratos aquosos de *Plectranthus*"

Introdução

Plantas com fins medicinais têm sido muito usadas durante um grande número de anos, e os seus extratos são ainda essenciais na terapia de hoje em dia, consistindo numa fonte de fármacos quase exclusiva da maioria da população mundial. As espécies de *Plectranthus* são usadas numa diversidade de situações. A sua utilização mais citada reside sobre as suas propriedades medicinais, como antissépticas, anti-inflamatórias e antimicrobianas. Embora várias plantas já tenham sido estudadas, a química de *Plectranthus* ainda é relativamente desconhecida. No entanto, sabe-se que os constituintes fitoquímicos maioritários deste género são os diterpenóides, os compostos fenólicos e os óleos essenciais. Este trabalho foca-se no estudo dos diterpenóides e dos compostos fenólicos. Os diterpenóides são os metabolitos secundários mais comummente encontrados nas espécies de *Plectranthus*, sendo a sua maioria abietanos altamente modificados. O principal composto fenólico encontrado é o ácido rosmarínico, já muito conhecido pelos seus interesses biológicos. Outros compostos fenólicos também muito encontrados nas espécies de *Plectranthus* são os flavonóides. Estes podem ser divididos em várias classes, sendo a maior classe a classe das flavonas. Neste trabalho, algumas flavonas são estudadas por espectrometria de massa.

A espectrometria de massa é provavelmente a técnica analítica mais versátil atualmente disponível para químicos. Mede exatamente a massa molecular de compostos individuais, através da ionização dos átomos ou moléculas de uma amostra, da sua separação pela razão massa/carga (*m/z*) e da sua deteção qualitativa e quantitativa pela respetiva *m/z* e abundância. Visto que, em princípio, todos os iões são acessíveis pela espectrometria de massa, é considerada um método universal para a análise química. As técnicas de espectrometria de massa são adequadas para a análise de flavonóides em plantas e alimentos, visto conseguirem fornecer informação estrutural significativa tanto em pequenas quantidades de amostras puras, como em misturas. A espectrometria de massa tandem com ionização por *electrospray* tem mostrado ser um método poderoso na identificação de compostos fenólicos em plantas. A espectrometria de massa tandem envolve pelo menos duas fases de análise de massa. Geralmente, um primeiro analisador de massa é usado para isolar o ião precursor, que depois sofre fragmentação por colisão com um gás inerte, originando iões produto e fragmentos neutros. Estes são então analisados por um segundo analisador de massa. Uma das principais vantagens da espectrometria de

massa tandem, para além da sua elevada especificidade, é o facto de a amostra não ter que sofrer uma purificação prévia, visto conseguir-se selecionar e isolar o ião de interesse.

Uma vez que as amostras de extratos de plantas são extremamente complexas, são necessários métodos analíticos seletivos e eficientes para determinar as estruturas dos seus compostos. A cromatografia líquida acoplada à espectrometria de massa tandem (HPLC-MS^n) permite a separação e identificação de moléculas individuais em amostras complexas, sendo, por isso, a escolha de eleição. Visto que os flavonóides são compostos moderadamente polares e termolábeis, são também melhor analisados por cromatografia líquida. Obviamente, e especialmente quando a identificação individual dos flavonóides é importante, são usadas técnicas hifenadas avançadas, como por exemplo o HPLC-MS^n).

Parte experimental

Todos os reagentes utilizados ao longo deste trabalho são de grau HPLC e não sofreram nenhuma purificação prévia.

As cinco flavonas estudadas (acacetina, chrysoeriol, luteolina, orientina e vitexina) foram dissolvidas em metanol a uma concentração de 10^{-5} M. As soluções estudadas no modo de ionização positivo foram acidificadas com 2 µL de ácido fórmico. Estas flavonas foram analisadas por espectrometria de massa e espectrometria de massa tandem utilizando, para tal, um espectrómetro de massa LCQ Duo da Thermo Scientific (USA). Os parâmetros instrumentais foram otimizados de modo a garantir a melhor razão sinal/ruído. Para as experiências tandem, o hélio foi usado com gás de colisão. Todos os dados foram adquiridos nos modos positivo e negativo, usando o *software* Xcalibur versão 1.2 da Thermo Scientific (USA).

Para prever os locais de protonação e desprotonação mais favoráveis para as cinco flavonas foram realizados cálculos semi-empíricos, usando para tal o PM6 hamiltoneano implementado no MOPAC2012. As entalpias de formação foram calculadas para cada composto, tanto para as espécies neutras como para as várias espécies protonadas e desprotonadas, sendo estas depois usadas para calcular as entalpias de reação.

Foram analisadas as folhas de dez espécies de *Plectranthus*. Depois de devidamente preparadas e liofilizadas, 10 mg de cada amostra foram diluídas em 1 mL de MeOH/H_2O (50/50), as amostras foram filtradas e introduzidas em vials.

As análises LC-MS^n foram realizadas com um HPLC Surveyor Plus da Thermo Scientific (USA) acoplado a um espectrómetro de massa LCQ Duo Thermo Scientific (USA)

equipado com uma fonte de ionização por *electrospray*. A coluna usada foi uma LiChrospher 100 RP-8 (5µm) LiChroCART 250-4 mm da Merck. Foram testados cinco métodos diferentes, diferindo entre eles a constituição do eluente, o fluxo e o tempo de análise. A fase móvel é constituída por um sistema binário de MeOH e uma fração aquosa. Estas foram: H_2O + 0.05% TFA, H_2O + 1% HCOOH, H_2O + 0.1% HCOOH e H_2O + 0.1% NH_4OH solução. Os parâmetros instrumentais foram otimizados de modo a garantir a melhor razão sinal/ruído. O hélio foi usado com gás de colisão. Todas as experiências foram realizadas nos modos positivo e negativo, e os espectros de massa foram adquiridos e processados usando o *software* Xcalibur versão 1.2 da Thermo Scientific (USA).

Resultados, discussão e conclusões

Através da realização de cálculos semi-empíricos verificou-se que a protonação é energeticamente mais favorável no grupo carbonilo (C=O) da posição 4 do anel C para as cinco flavonas. Quanto à desprotonação, esta é mais provável ocorrer no grupo hidroxilo (OH) da posição 7 para a acacetina, no grupo OH da posição 4' para o chrysoeriol e para a luteolina, e no grupo OH da posição 4 do açúcar para a orientina e para a vitexina.

As cinco flavonas foram estudadas por espectrometria de massa com ionização por *electrospray* nos modos positivo e negativo. Relativamente ao modo negativo, foram verificadas perdas de H_2O, CO, CO_2, CH_3 e C_2H_2O, juntamente com quebras de ligações C-C no anel C da aglícona e no açúcar, quando aplicável. Foram apresentados alguns exemplos destas várias vias de fragmentação. Na análise em modo positivo, verificaram-se perdas de H_2O, CO e C_2H_2O, juntamente com quebras de ligações C-C no anel C da aglícona e no açúcar, quando aplicável. Para estes casos também foram apresentadas algumas vias de fragmentação exemplificativas. Os resultados analisados sugerem que os dois modos de ionização são complementares na caracterização estrutural de flavonas por espectrometria de massa.

Vários métodos foram testados para a separação e identificação dos compostos fenólicos e dos diterpenóides presentes nos extratos de *Plectranthus*. Todos os métodos de HPLC iniciavam com uma maior percentagem da fase aquosa, e no final de cada método as condições iniciais eram restabelecidas. A otimização das condições cromatográficas incluiu a constituição do eluente, o fluxo e o tempo de análise. Vários problemas foram encontrados, como por exemplo a formação de adutos entre os compostos e o eluente ou então a baixa resolução dos picos. No final, chegou-se à conclusão de que as condições cromatográficas

usadas para a identificação dos compostos seriam as seguintes: corridas de 90 minutos, um fluxo de 0.3 mL min^{-1}, e a fase móvel seria composta por um sistema binário de MeOH e H_2O + HCOOH 0.1%.

A identificação dos compostos presentes em cada amostra foi feita através da análise das suas fragmentações. Vários compostos foram identificados nos dez extratos, sendo que o ácido rosmarínico (conhecido pelas suas propriedades antioxidantes) foi o único encontrado em todas as amostras e o presente em maior abundância. A percentagem de ácido rosmarínico presente nas amostras foi depois comparada com o potencial antioxidante. Contudo, alguns compostos ficaram por identificar.

Abbreviations and Symbols

a.u. – arbitrary unit

CI – chemical ionization

CID – collision induced dissociation

CRM – charge residue model

DFT – density functional theory

ECD – electron capture dissociation

EI – electron ionization

ESI – electrospray ionization

ESI-MS/MS – electrospray ionization tandem mass spectrometry

ETD – electron transfer dissociation

EtOAc – ethyl acetate

FAB – fast atom bombardment

FTICR – Fourier transform ion cyclotron resonance

GC – gas chromatography

HCOOH – formic acid

HPLC – high performance liquid chromatography

H_2O – water

ICR – ion cyclotron resonance

IC50 – half maximal inhibitory concentration

IEM – ion evaporation model

IRMPD – infrared multiphoton dissociation

IT – ion trap

kDa – kilodalton

kJ – kilojoules

kV – kilovolts

LC – liquid chromatography

LC-MS – liquid chromatography-mass spectrometry

LC-MS/MS – liquid chromatography-tandem mass spectrometry

MALDI – matrix assisted laser desorption/ionization

Me – methyl

MeOH – methanol

m_f^+ - product ion

m_n – neutral fragment

mol – mole

m_p^+ - precursor ion

MS – mass spectrometry

MS^n – Multi stage mass spectrometry (n – number of generations of ions being analysed)

m/z – mass-to-charge ratio

NaCl – sodium chloride

Na^+ – sodium ion

NH_4OAc – ammonium acetate

NH_4OH – ammonium hydroxide

NH_4^+ – ammonium ion

PA – *Plectranthus apimentado*

PE – *Plectranthus ersti*

PG – *Plectranthus gradientatus*

PL – *Plectranthus lanuginosus*

PM – *Plectranthus madagascariensis*

PN – *Plectranthus neochilus*

PV – *Plectranthus verticilatus*

PVEN – *Plectranthus ventrii*

PVUB – *Plectranthus verticilatus u. bombo*

PZ – *Plectranthus zuluensis*

RA – rosmarinic acid

RDA – retro-Diels-Alder

SID – surface induced dissociation

SPE – solid phase extraction

TFA – trifluoroacetic acid

t_m – time required for the mobile phase or an unretained solute to pass through the chromatographic column

TOF – time-of-flight

torr – torr

t_R – retention time

t'_r – adjusted retention time

UV – ultraviolet

V – volts

α – selectivity

$\Delta_f H$ – heat of formation

$\Delta_r H$ – reaction enthalpy

µl – microlitre

General Contents

Figure List

Table List

CHAPTER 1

INTRODUCTION

1.1. The Importance of Chemical Analysis in Food Stuff

In the 18th and 19th centuries, the major aim for the chemists was to understand the chemical nature of food. They realised that this knowledge was essential if dietary standards were to improve. With them also health and prosperity would improve. Obviously, the food components present in larger amounts, such as carbohydrates, were the first nutrients described by chemists. As it was known that food and drink on sale to the general public could have been adulterated, it was necessary to develop analytical techniques that allow more selectivity and sensitivity to identify compounds in lower concentrations (for example vitamins). These analytical procedures (for example, gas chromatography, high-performance liquid chromatography and mass spectrometry) are used to provide information about a wide variety of different characteristics of foods, including their composition, structure, physicochemical properties and sensory attributes. It was in the 20th century with these techniques that chemists detected malpractices in food suppliers, such as additional water in milk, dangerous colourings in confectionary, amongst others.

Nowadays, the knowledge of chemical analysis has become important not only for scientists in their research, but also bears influence in the human beings' daily routine as well. The information about the food stuff is critical to the rational understanding of the factors that determine the properties of foods, as well as to the ability to economically produce foods that are consistently safe, nutritious and desirable and for consumers to make informed choices about their diet.[1]

1.2. Plant extracts: *Plectranthus*

Plants for medicinal purposes have been in use for a great number of years, and their extracts are still essential to today's therapy, consisting in an almost exclusive source of drugs for the majority of the world population.[2]

Figure 1.1 – Two different *Plectranthus* species (family Lamiaceae, subfamily Nepetoideae, tribe Ocimeae).

Many *Plectranthus* species are plants with economic potential and medicinal interest. The genus *Plectranthus*, a large and widespread genus containing around 300 species, belongs to the family Lamiaceae, subfamily Nepetoideae, tribe Ocimeae. They are warm-climate plants that occur largely in the southern hemisphere.

Plectranthus species are used in a variety of situations. The most cited use is for their medicinal properties, such as antiseptic, anti-inflammatory and antimicrobial. As example, *P. barbatus* is used as a remedy for stomach ache and as a purgative.[3, 4]

1.2.1. Chemical constituents isolated from *Plectranthus*

Although several plants have already been studied, the chemistry of *Plectranthus* is still relatively unknown. To date, the majority of the phytochemical studies on this genus have focused on the isolation of a range of diterpenoids.

The main phytochemical constituents of *Plectranthus* genus are diterpenoids, phenolics and essential oils. In this work, the focus remains on the diterpenoids and the phenolic compounds (structure depicted in **Figure 1.2**).[4, 5]

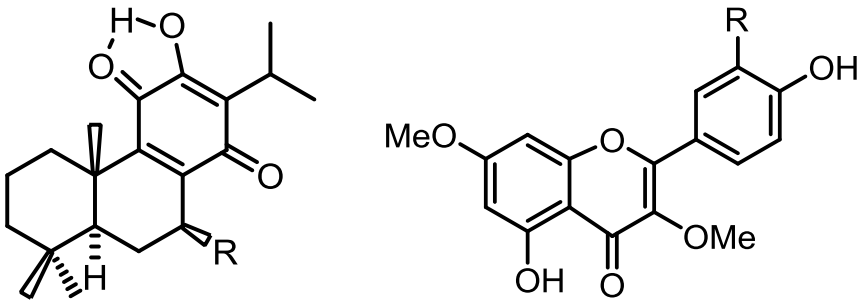


Figure 1.2 – Example structures of a diterpenoid and a phenolic compound (flavone) found in Plectranthus species. (Adapted from references [3] and [5])

1.2.1.1. Diterpenoids

Diterpenoids belong to a class of naturally occurring secondary metabolites that present a large variety of structures (as can be seen in **Figure 1.3**). The diterpenes contain four isoprene units, possessing 20 C-atoms and four branched methyl (Me) groups.

Diterpenoids are found mainly in Angiospermae, and amongst Angiospermae, Lamiaceae is one of the families where they are isolated from the most. They are the most common secondary metabolites found in *Plectranthus* species, being generally identified from the coloured leaf-glands. The majority of them are highly modified abietanoids.[3, 5]

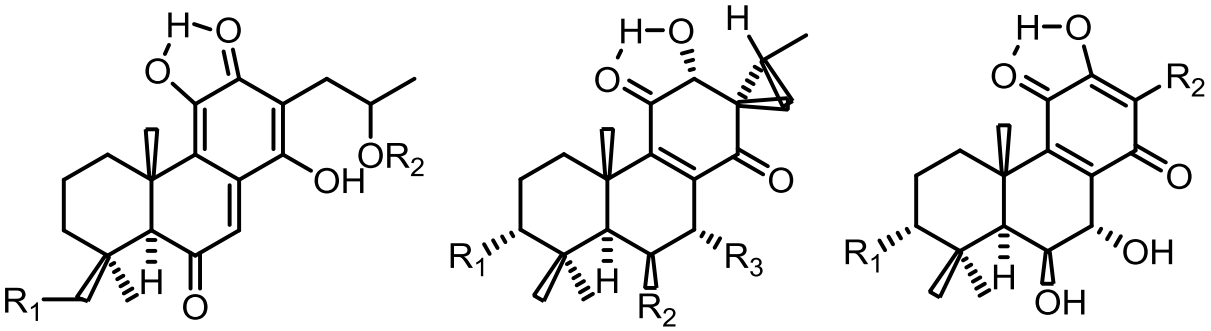


Figure 1.3 – Three examples of the variety of structures that diterpenoids can assume. (Adapted from reference [3])

1.2.1.2. Phenolic compounds

The main compound found in polar extracts from *Plectranthus* is rosmarinic acid (RA), which is a common component within the subfamily Nepetoideae of the Lamiaceae family. Rosmarinic acid (**Figure 1.4**) is an ester of caffeic acid and 3,4-dihydroxyphenyl lactic acid. The presence of this caffeic acid ester and also of others, like nepetoidin A (**Figure 1.4**), permits to distinguish this subfamily. Rosmarinic acid possesses a large variety of biological

interests, such as antiviral, antibacterial and antioxidant effects. Thus, its presence in medicinal plants, herbs and spices is beneficial to human health.

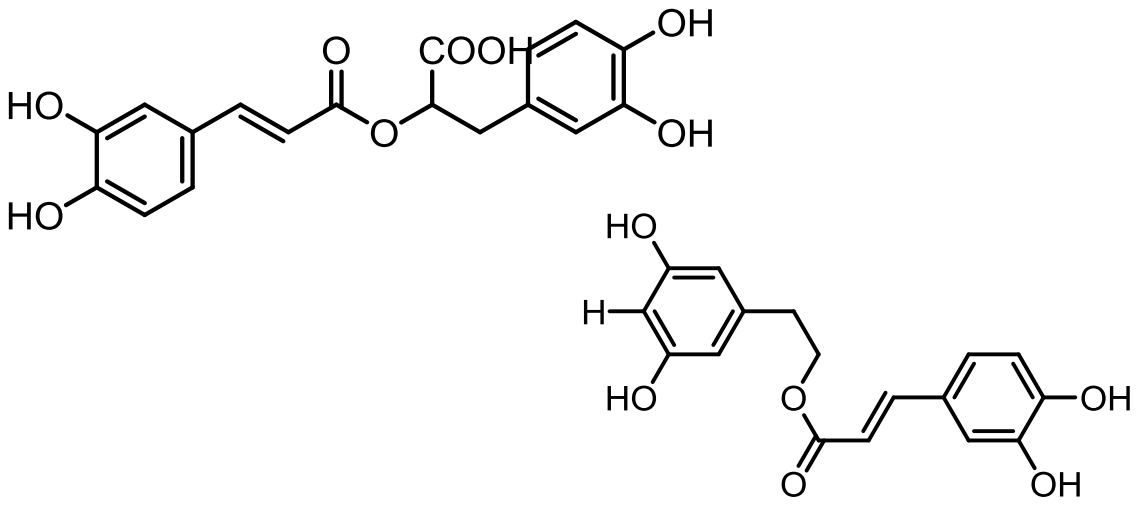


Figure 1.4 – Chemical structures of rosmarinic acid (above) and nepetoidin A (below). (Adapted from references [6] and [7])

Other phenolic compounds that have also been found in *Plectranthus* species are flavonoids. These compounds will be described in the next section.[5-7]

1.3. Flavonoids

"…Areas dedicated to the cultivation of tea have a low index of all types of cancer…"

MEDLINE

About 2% of all carbon photosynthesized by plants is estimated to be converted into flavonoids or related compounds. The list of flavonoids that have already been described reports more than 5000, but it is constantly expanding.[8-10]

1.3.1. Occurrence and Properties

Flavonoids are considered almost universal pigments of plants; they are a large group of phytochemicals mainly found in the petals, the foliage of trees and bushes, being widely distributed in the edible parts of the plants, providing protection against pathogens and herbivores. Although free aglycones have been found in a variety of plants, flavonoids are generally present as *O*- and/or *C*-glycosides.[8, 11, 12]

For the plants, flavonoids are responsible for the colour, flavour and aroma of fruits and flowers (**Figure 1.5**); they also act as enzyme inhibitors, as a defence against insects and ultraviolet (UV) radiation exposure, as chelating agents of metals that are noxious to plants, amongst others.[9, 13]

Figure 1.5 – Colour of flowers and fruits due to the presence of flavonoids.

However, the interest mainly remains in the physiological actions of flavonoids and their beneficial effects on human health. The best known property of flavonoids is their ability to act as antioxidants, by interfering with at least three different free radical-producing systems: they are able to reduce highly oxidizing free radicals, to scavenge nitric oxide, and also to inhibit xanthine oxidase. Due to these antioxidant properties, a lot of

flavonoids possess biological activities such as anti-inflammatory, anti-allergic and anti-tumoral. They have been found to be an important constituent of the human diet, being consumed in fresh fruits and vegetables or processed products (**Figure 1.6**), and are considered to be active principles of various medicinal plants. They are regularly used for the prevention of cancer, dementia, atherosclerosis and coronary heart disease. Thus, flavonoids present in foodstuffs and nutraceuticals have received much attention over the years.[8, 9, 14]

Figure 1.6 – Some foodstuff where flavonoids have been found.

1.3.2. Chemical Structure

All flavonoids present the general structure of a 15-carbon skeleton (aglycone) that consists of two phenyl rings and a heterocyclic ring. According to the differences in the functional groups and their relative position of the aglycone, flavonoids can be divided into several classes, such as isoflavones, flavones, flavonols, amongst others (**Figure 1.7**).[9, 15]

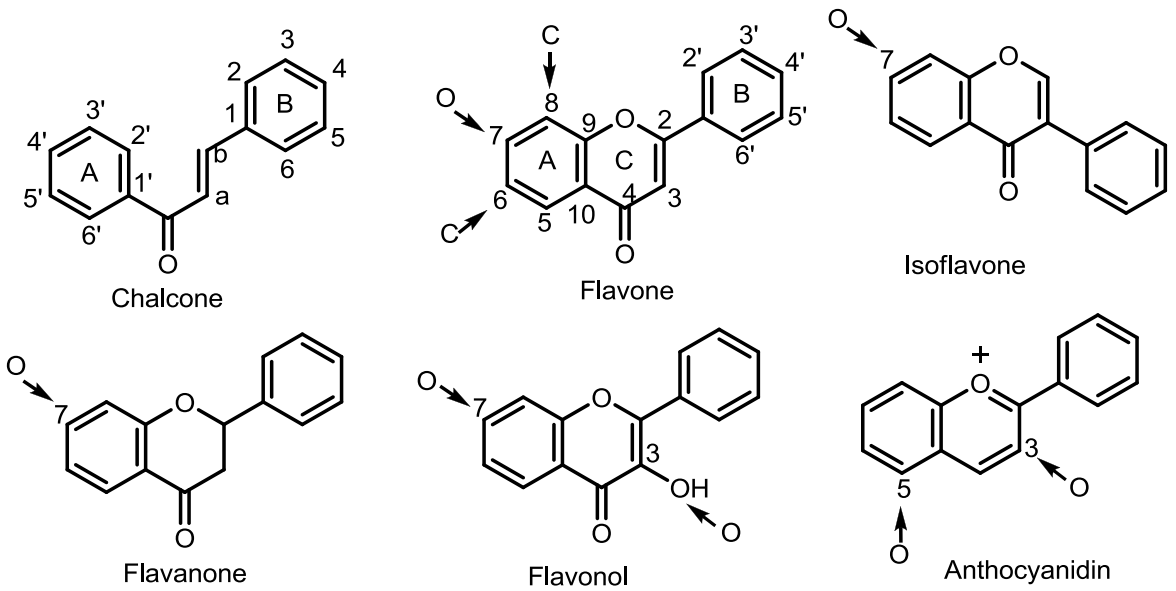


Figure 1.7 – Basic structures corresponding to the main classes of flavonoids, with the common *O*- and *C*-glycosylation positions indicated with an arrow. (Adapted from reference [9])

1.4. Mass Spectrometry (MS)

Mass spectrometry (MS) is probably the most versatile analytical technique currently available for chemists. It measures precisely the molecular masses of individual compounds, by ionising the atoms or molecules of the sample, separating them by their *mass-to-charge* (*m/z*) ratio and detecting them qualitatively and quantitatively by their respective *m/z* and abundance. Since, in principle, all atomic and molecular ions are accessible by MS, it is considered a universal method for chemical analysis.[16-18]

Mass spectrometric techniques are suitable to analyse flavonoids in plants and foodstuffs, since they can provide significant structural information on small quantities of pure samples as well as on mixtures, and ESI-MS/MS has been shown to be a powerful method for the identification of plant phenolics.[9, 19]

1.4.1. Brief History and Recent Developments

To really appreciate the way that the mass spectrometry field has expanded until the present, it is useful to look back and examine some of the great advances and the people who made them happen.

Although MS is mostly under the purview of analytical chemistry, it was born in the physics field.[20] The birth of MS occurred in 1897 with the studies of Sir J. J. Thomson, studies these that in 1907 led to the construction of a parabola mass spectrograph (first mass spectrometer).[21, 22] In 1906, Thomson received the Nobel Prize in Physics for his work in "discovering" the electron and determining its *m/z* ratio.[20] As a continuation to his investigations, Thomson and his protégé Francis W. Aston built the first mass spectrometer with velocity focusing. In the first three decades of the 20th century, Aston redesigned the instruments, improving their resolving power and began to use them to separate and prove the existence of elemental isotopes.[23] These studies with isotopes won him his own Nobel Prize in Chemistry, in 1922.[20]

A large contribution to the development of mass spectrometers was the introduction of the electron ionization (EI) source for solids by A. J. Dempster, in 1918[24], and for gases by W. Bleakney, in 1929[25]. Nowadays, this ionization source is used in large scale coupled to gas chromatography (GC).

In the 1940s, chemists recognised MS as a technique with great potential in analytical chemistry, and the first commercial mass spectrometer became available in 1943.

The time-of-flight (TOF) mass analyser and the ion cyclotron resonance (ICR) were introduced in 1946 and in 1949, respectively.[26, 27] In 1953, W. Paul described the quadrupole mass analyser and the ion traps.[28]

The 1960s witnessed the development of tandem MS and collision-induced decompositions[29], being very important in the field of structural analysis. But, in spite of all these improvements, the analysis of molecules of higher molecular weight was not yet possible. The first significant progress occurred in 1966, by M. S. Munson and F. H. Field, who introduced the chemical ionization (CI) technique, allowing the ionization of labile biomolecules.[30]

During the 1980s, a variety of softer ionizations methods were developed to try to solve the problem of the larger molecules. The first technique that had a great impact was fast atom bombardment (FAB), introduced by M. Barber in 1981.[31] Then, matrix-assisted laser desorption ionization - MALDI - (developed by F. Hillenkamp and M. Karas, in 1987)[32] and electrospray - ESI - (developed by J. Fenn, 1988)[33] appeared almost simultaneously. These ionization techniques revolutionized biological MS (the upper mass range was extended beyond 100 kDa) and are still the elected ones for macromolecule ionization.[20] Fenn also demonstrated that is was possible to obtain accuracy in the measurement of molecular weight, by applying a method that accounts the average of the obtained signals for the multiple ions formed during the electrospray process. Fenn, for his work on ESI, and Tanaka, for his laser desorption method of protein ionization, both won the Nobel Prize in Chemistry in 2002.[17, 20, 34, 35]

Recently, we have witnessed new developments in the MS field, such as the introduction of two new ion traps, the orbitrap in 2000, by A. Makarov,[36] and the linear ion trap in 2002, by J. W. Hager.[37] Also recently, a novel interface adapter for the EI technique was developed, allowing a new way of direct coupling to a nano-liquid chromatograph.[38]

1.4.2. The Mass Spectrometer

Typically, a mass spectrometer consists of five components: an inlet system, an ion source, a mass analyser, an ion detector and a data system (as shown in **Figure 1.8**).

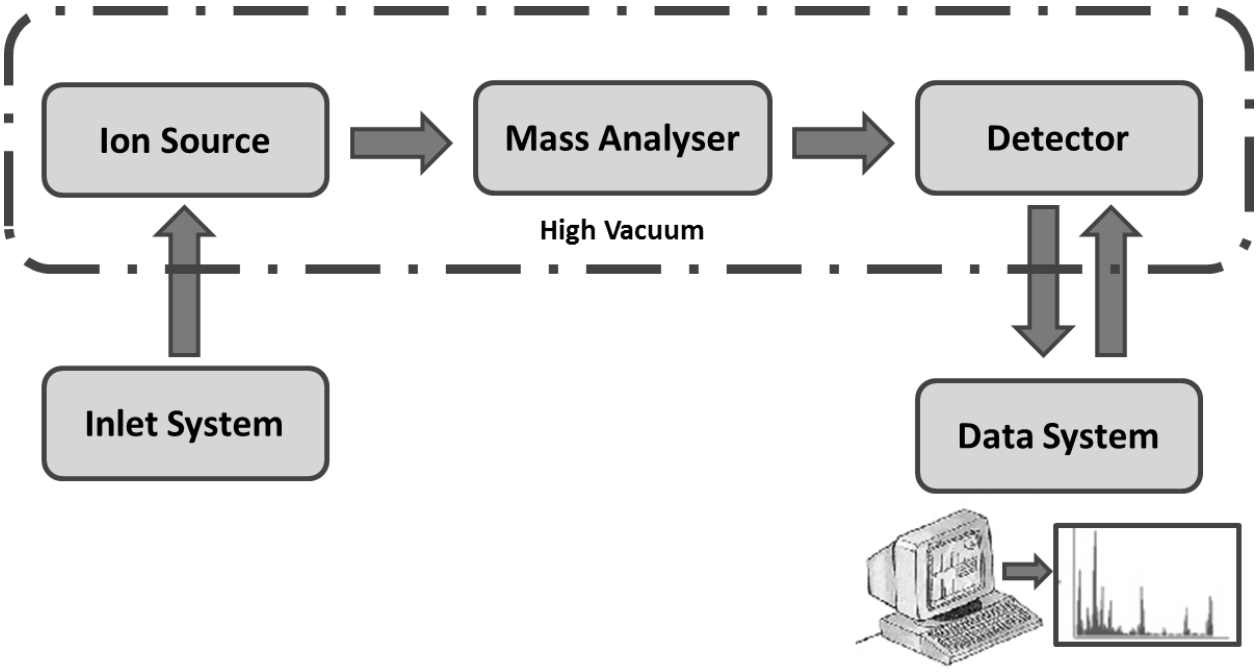


Figure 1.8 – The five main components of a mass spectrometer. (Adapted from reference [34])

Through the inlet system, a sample is introduced into the mass spectrometer and transferred into the gas phase. The gas phase analytes are then ionized in the ion source and transferred into the mass analyser. It is in the mass analyser that the ions are separated according to their *m/z* ratio. Except for the FTICR and orbitrap analysers, which also work as a detector, the ion detection is accomplished by an electron multiplier system, where the ion current is multiplied by acceleration of electrons on the surface of an electrode, which originates secondary electrons. This way, by means of an electric signal, the information reaches the data system, which exhibits it as an appropriate mass spectrum. The data system also controls the instrument through feedback.[34, 39]

The MS process (from the ion source to the detector) is carried out under high vacuum. To maintain this vacuum, all mass spectrometers are equipped with a vacuum system. This is necessary to avoid collisions between the ions and other gaseous molecules during their course until reaching the detector. If these collisions occurred, the ions would lose their charge against the walls of the instrument. The high vacuum requires two pumping stages: the first one is a mechanical pump (rotary pump) that provides rough vacuum down to 10^{-3} torr; the second one uses turbomolecular pumps or diffusion pumps

to provide high vacuum (10^{-5} torr). ICR instruments often include a cryogenic pump for a third pumping stage, due to its even higher vacuum requirements.[39, 40]

1.4.2.1. Ion Source

There is a variety of ionization techniques that can be used in MS. The most important considerations to take into account when making a choice are the internal energy transferred during the ionization process and the physico-chemical properties of the analyte that is going to be analysed. Within these techniques, the most common ones are EI, CI, MALDI and ESI.

In this work, the mass spectrometer used was equipped with an ESI ion source.

1.4.2.1.1. Electrospray (ESI)

The ESI process was first described in 1968 by M. Dole *et al*,[41] which recognised the possibility of generating gas-phase ions of macromolecules by spraying a solution from the tip of an electrically charged capillary. About two decades later, J. Fenn and co-workers developed ESI as a true interface for MS.[33]

The ESI process (described in **Figure 1.9**) is generally divided into three main steps: **charged droplet formation, droplet shrinkage and gaseous ion formation**. A diluted solution of the analyte is passed through a capillary tube with a weak flux (1-10 µl min^{-1}), which is held at high voltage. This high voltage creates an electric field gradient between the capillary and the counter electrode, which induces a charge separation at the liquid surface. Due to surface destabilization the liquid is projected, assuming a cone shape, known as the Taylor Cone (named after Sir G. Taylor). When the liquid that comprises the Taylor cone reaches the Rayleigh stability limit (point when the Coulombic repulsions of the surface charge equal the surface tension of the liquid), droplets that contain an excess of (positive or negative) charge detach from its tip. Then, these droplets move through the atmosphere towards the entrance of the mass spectrometer, and generate gaseous ions by one of the possible mechanisms.[17, 18, 42-44]

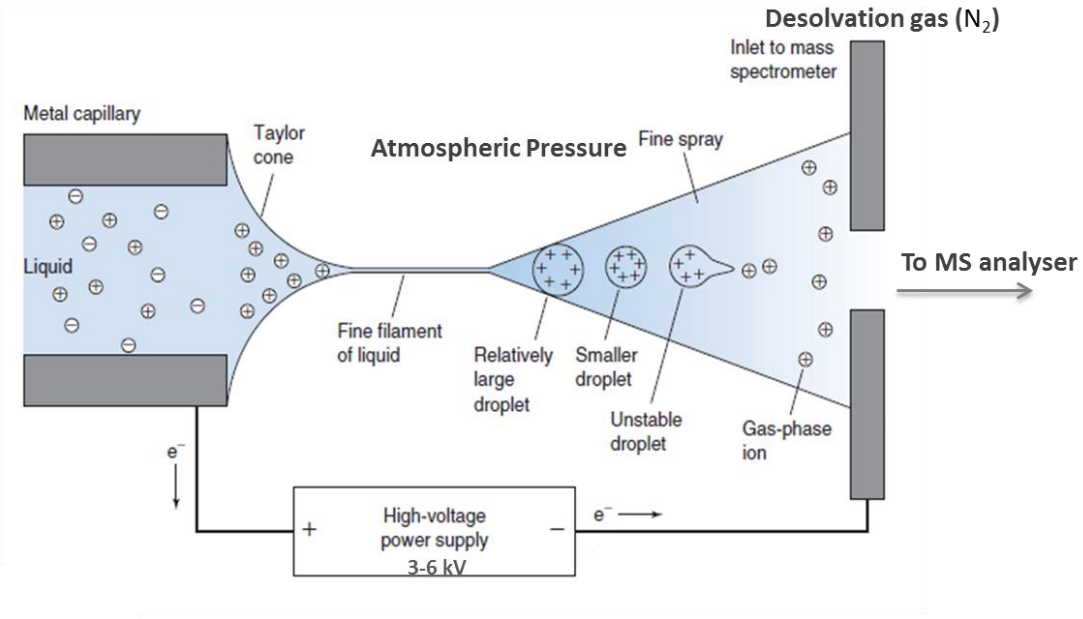


Figure 1.9 – The different stages of the ESI process. (Adapted from reference [45])

There are mainly two possible mechanisms for the production of gas-phase ions in ESI (**Figure 1.10**). These are the Charge Residue Model (CRM), proposed by M. Dole In 1968, and the Ion Evaporation Model (IEM), proposed by Iribarne and Thomson in 1979. It is considered that these two models describe the two extremes of the same general process; while the IEM prevails for relatively small ions (m/z < 3300), the CRM seems to be fit for larger multiply charges species.

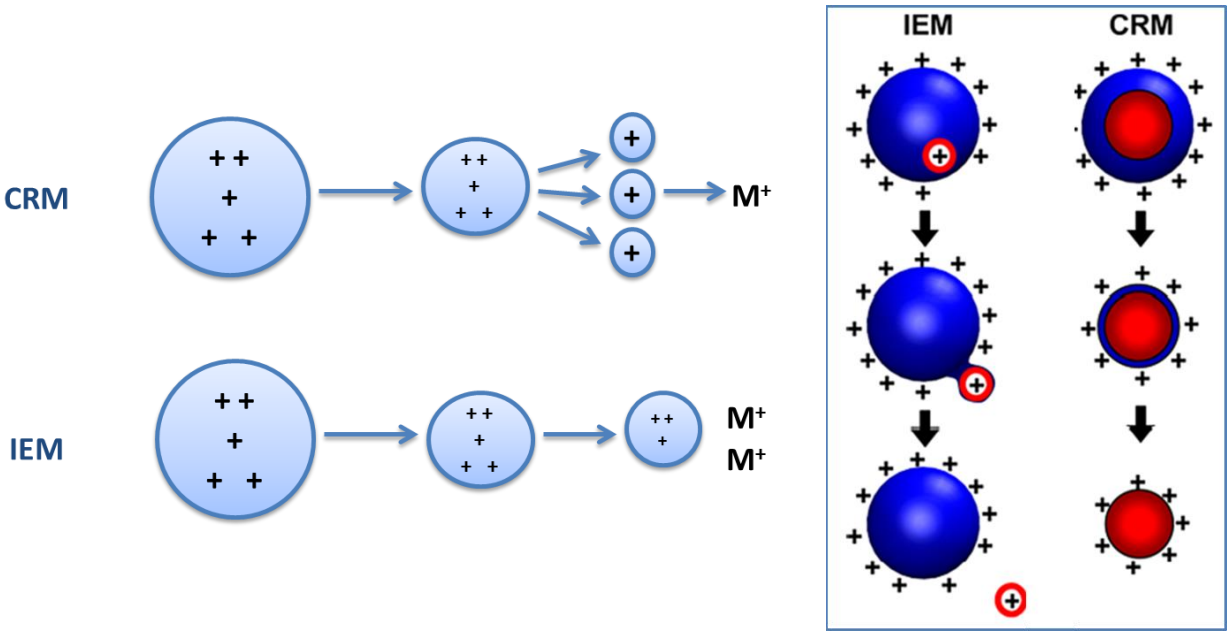


Figure 1.10 – The two possible mechanisms for the production of gas-phase ions in ESI (CRM and IEM). (Adapted from reference [44])

By CRM, solvent evaporation from the charged droplets leads to an increase of electrostatic repulsions at the surface. This causes the droplets to undergo a Rayleigh explosion, originating smaller droplets. Successive divisions result in the production of mono or multicharged droplets that contain only one molecule of the analyte. The IEM predicts that direct ion emission occurs after the droplets suffer shrinkage by solvent evaporation. Recently, J. H. Gross and co-workers suggested a modification of the CRM, in which CRM is preceded by IEM. Regardless of the mechanism by which they are produced, it is certain that the ESI process generates vapour phase ions that can be analysed by their *m/z* within a mass spectrometer.[39, 42-44, 46]

Octopoles, quadrupoles and lenses are used to transfer the ions from a higher pressure region to a lower pressure region. A nebulizing and drying gas (usually nitrogen) is used to assist in the droplets desolvation process and to prevent the formation of agglomerates or clusters throughout the capillary. The sampling of the fully or partially desolvated ions is made using a capillary or a skimmer device. Skimmers are cones with a small aperture (**Figure 1.11**) which enable to establish a pressure gradient between the ion source and the mass analyser. The skimmers also acts as a time separator, separating the ions from the sample (heavier) from the molecules (lighter), the solvent and the gas.[42, 47]

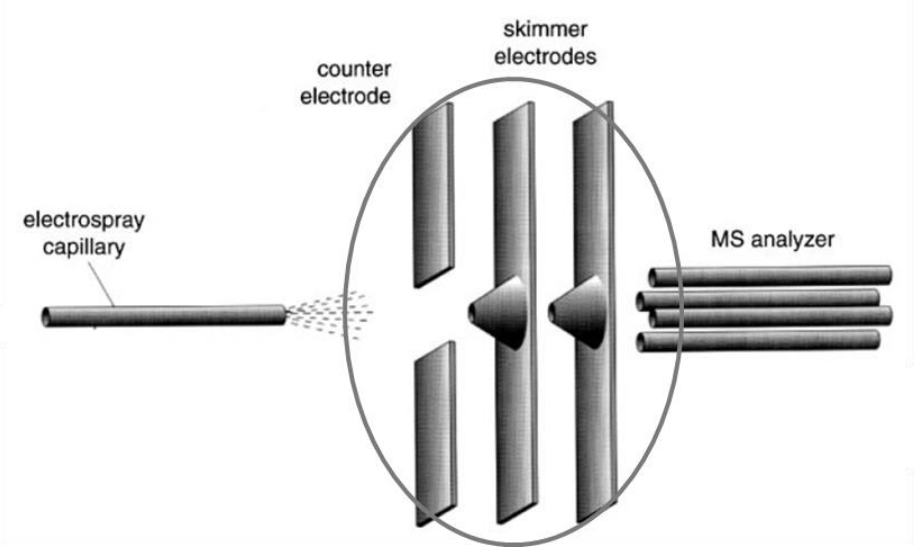


Figure 1.11 – Representation of a skimmer device. (Adapted from reference [42])

1.4.2.2. Mass Analyser

There are several different types of mass analysers that can be used in MS: sector instruments, quadrupoles and ion traps, time-of-flight, Fourier transform ion cyclotron resonance and *orbitraps*.

In this work, the mass spectrometer used was equipped with an ion trap type mass analyser, in which a radiofrequency electric field allows the separation of the ions according to their *m/z* ratio.

1.4.2.2.1. Ion Trap (IT)

Paul and Steinwedel described an IT in 1960[48, 49], and it was modified to a useful mass spectrometer by Stafford *et al.* in 1984.[50]

The IT mass analyser is formed by two endcap electrodes, which present a hyperbolic geometry, and a ring electrode, positioned in the centre (as can be seen in **Figure 1.12**).

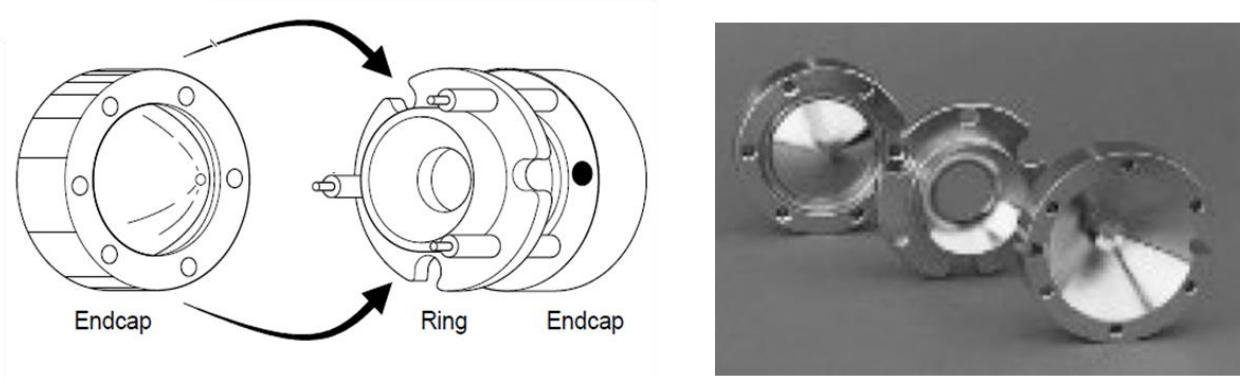


Figure 1.12 – Components of an IT mass analyser. (Adapted from references [16] and [45])

As shown in **Figure 1.13**, in this type of analyser, a three dimensional field is created, which confines the ions to the middle of the analyser. This field generates stable trajectories for ions of a certain *m/z* range, which are sequentially ejected, in direction to the detector. While doing this, it also removes unwanted ions by colliding them with the walls of the IT. Therefore, the mass separation in the IT is achieved by storing the ions in the trapping space and manipulating their motion in time, rather than in space.

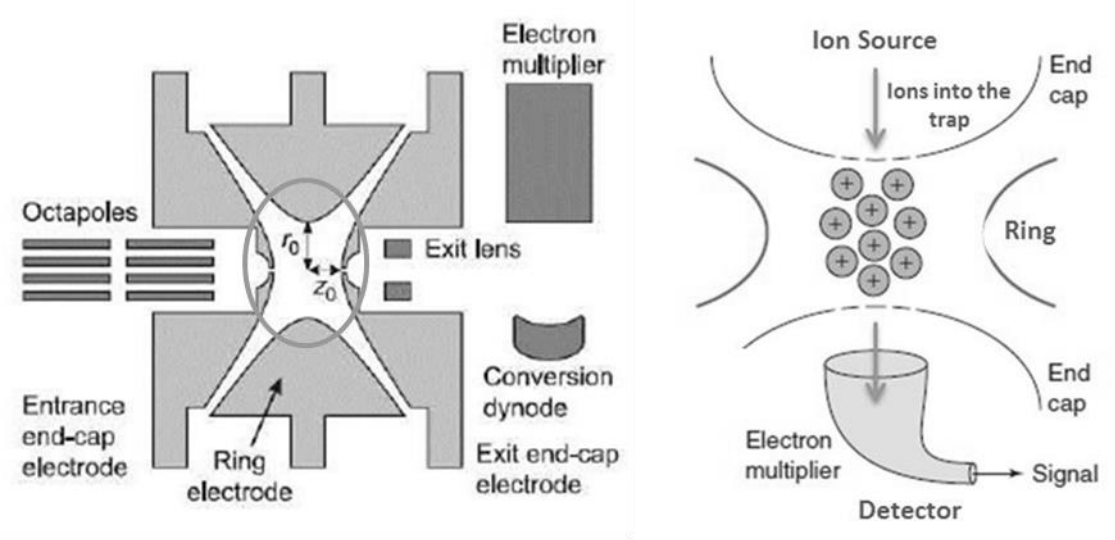


Figure 1.13 – Trajectory of the ions trough the ion trap. (Adapted from references [39] and [45])

In chemical analysis, the principle advantages of the IT can be summarized as follows:

- High sensitivity;
- Relatively inexpensive;
- Compactness and mechanical simplicity in a device capable of high performance;
- Enables tandem mass spectrometry experiments by performing sequential mass analysis measurements;
- Ion/molecule reactions can be studied for mass-selected ions.[16, 34, 40, 45]

1.4.3. Tandem Mass Spectrometry (MS^n)

Tandem MS involves at least two stages of mass analysis, either in conjunction with a dissociation process or in a chemical reaction that causes a change in the mass or charge of an ion. Generally, a first mass analyser is used to isolate the precursor ion (m_p^+), which then suffers fragmentation to yield product ions (m_f^+) and neutral fragments (m_n) that are then analysed by a second mass analyser:

$$m_p^+ \longrightarrow m_f^+ + m_n$$

An activation barrier must be overtaken so this reaction can occur. The energy required for such process can come from one of two sources:

I. From the excess energy deposited on the precursor ion by the ionization process. This is valid only when dealing with electron ionization at high energies.

II. From activation methods such as collision induced dissociation (CID), infrared multiphoton dissociation (IRMPD), electron capture/electron transfer dissociation (ECD/ETD) and surface induced dissociation (SID).[17]

The principle of tandem MS is shown in the figure below (**Figure 1.14**). A precursor ion is isolated by the first mass analyser, fragmented by collision with an inert gas and the product ions are then analysed by the second mass spectrometer.

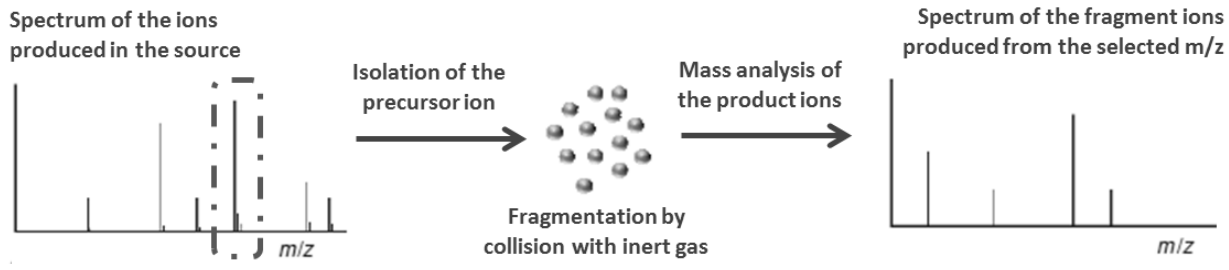


Figure 1.14 – The principle behind tandem MS. (Adapted from reference [17])

MS^3 experiments are possible to perform by increasing the number of steps: select ions of a first mass, then select ions of a second mass from the fragments, obtain and finally analyse the fragments of these last selected ions. The number of steps can be increased further to yield an MS^n experiment. Nevertheless, this will certainly increase the complexity of the instrument and consequently its cost.

Tandem MS can be conceived in two ways: in space, by the coupling of two mass spectrometers (quadrupole type analysers and TOF); or in time, by performing an appropriate sequence of events in an ion storage device (IT and ICR). In this process, the ion activation step is crucial and defines the type of product ions that are observed. There are several activation methods that can be used. In this work, the tandem MS experiments were performed by CID, to enable structural information of the compounds.

CID is still the most common ion activation method available. The ion activation is achieved by collisions between the precursor ion and a neutral gas target (helium), which is accompanied by an increase in the internal energy. This increase leads to the decomposition of the ion. In an IT, the CID occurs actually in the trap, which works as collision chamber. The

sample ions are accelerated and collide with the background helium gas, which leads to their fragmentation.

One of the main advantages of tandem MS, in addition with its enhanced specificity, is that the sample does not need to undergo a prior purification, because one is able to select and isolate the ion of interest.[17, 34, 51, 52]

I feel sure that there are many problems in Chemistry which could be solved with far greater ease by this than by any other method. The method is surprisingly sensitive – more so even than that of spectrum analysis – requires an infinitesimal amount of material and does not require this to be specially purified…

(Thomson 1921)

1.5. High-Performance Liquid Chromatography (HPLC)

Since the components of herb samples are very complex, selective and efficient analytical methods are required to determine their structures. HPLC-MS/MS allows the separation and identification of individual molecules in complex samples, being the general choice.

How flavonoids are moderately polar, thermolabile compounds, they are best analysed with HPLC. Obviously, and especially if identification of individual flavonoids is important, advanced hyphenated techniques are used (HPLC-MS or HPLC-MS/MS).[19, 51]

Liquid chromatography (LC) was defined in the early 1900s through the work of the Russian botanist M. S. Tswett. His studies involved separating compounds (leaf pigments), extracted from plants using a solvent, in a column packed with particles. He observed different coloured bands separating throughout the column. Tswett then coined *chromatography* to describe his experiment.[53] In the 1960s HPLC was developed as an analytical tool, being in these days one of the most powerful ones in analytical chemistry.[54, 55]

1.5.1. The HPLC System

A modern HPLC system, as presented in **Figure 1.15**, is generally composed by a solvent delivery system, a sample injection valve, a high-pressure column, a detector, and a computer, which controls the system and displays the results. Many systems also include an oven that controls the temperature of the column. As columns are expensive and easily degraded, some equipment possess a short guard column at the entrance of the main column, which protects it from impurities that could reduce its durability.[45, 56]

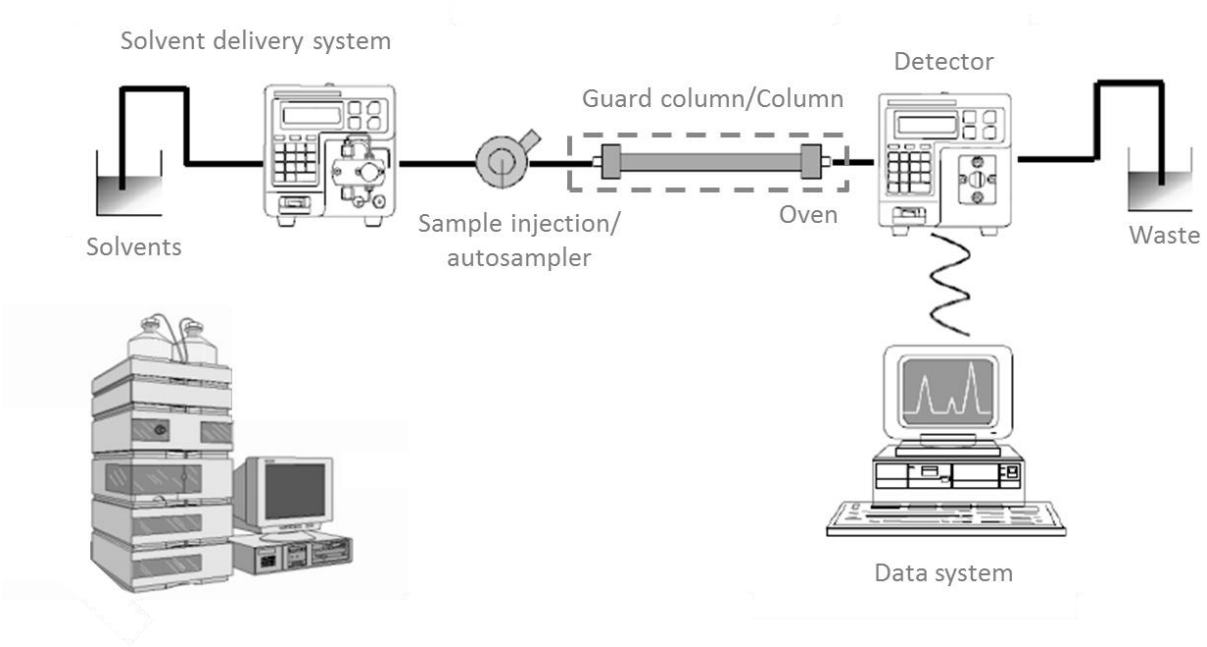


Figure 1.15 – Components of a typical HPLC system.

1.5.2. The Chromatographic Process

The successful use of HPLC requires the right combination of operating conditions, such as the separating temperature, the type of mobile phase and its flow rate, and so on. To select these best conditions it is important to understand various factors that control the HPLC separation.

Differential migration constitutes the basis of chromatography separation. It results from the equilibrium distribution of the different compounds between the stationary phase (fixed to a column or surface) and the mobile phase (that in HPLC is liquid). Normal-phase chromatography uses a polar stationary phase and a less polar solvent. On the other hand, in reversed-phase chromatography the stationary-phase is nonpolar or weakly polar and the solvent is more polar. The choice of these phases is made based on the type of sample and the compounds to be analysed. Also based on these factors is the choice of the type of elution (isocratic elution or gradient elution). Isocratic elution is performed with a constant

composition of eluent over time, while in gradient elution the composition of the eluent varies with time.

As shown in **Figure 1.16**, the compounds pass through the column according to their affinity for both phases, and as they reach the end of the column they are carried off to the detected and their concentrations are recorded in function of time, which is presented in form of a chromatogram.

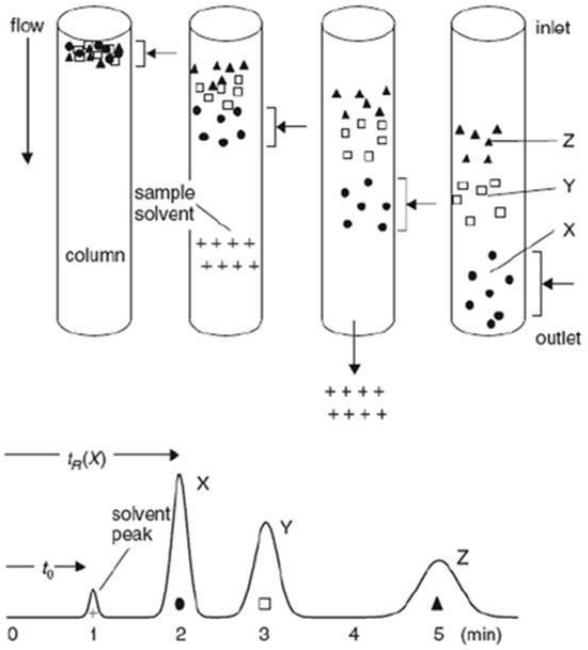


Figure 1.16 – Representation of the chromatographic process. (Adapted from reference [45])

Each peak of the chromatogram emerges from the column at a characteristic time, which can be used to identify the compounds. The retention time (t_R) is measured since the time the sample is injected to the time the first peak maximum leaves the column. The difference between the retention time and the time required for the mobile phase or an unretained solute to pass through the column (t_m) is denominated adjusted retention time (t'_r) (**Equation 1.1**).

Equation 1.1 $$t'_r = t_R - t_m$$

Another important feature in chromatography is the selectivity (α) (**Equation 1.2**), in other words, the capability of an analytical method to distinguish an analyte from other species in the sample. The selectivity between two peaks is determined by the ratio between the adjusted retention times of the two compounds. The higher the selectivity is, better will be the separation between the two components.

Equation 1.2 $$\alpha = t'_{r1} / t'_{r2}$$

The efficiency, also important in chromatography, consists in the column's capacity to limit the spreading of the peaks and therefore offer higher resolution. Thus, the straighter the peaks are, higher the resolution, so better the separation between two compounds. The efficiency of the column is generally measured by the number (N) (**Equation 1.3**) and height (H) (**Equation 1.4**) of the theoretical plates. Smaller the height of the plates, straighter the peaks are. So, in other words, for the column to reach maximum efficiency it should have a large number of theoretical plates with small height.[45, 56]

Equation 1.3 $$\mathbf{N = 16 * \left(\frac{t_R}{W}\right)^2}$$

N: number of theoretical plates;

t_R: retention time;

W: peak width.

Equation 1.4 $$\mathbf{H = L / N}$$

H: height equivalent to a theoretical plate;

L: column length;

N: number of theoretical plates.

1.5.3. LC-MS Interface

The development of coupled instrumental systems such as LC-MS helped to overcome some disadvantages of the manual methods and allowed the qualitative identification of unknown peaks. Several methods have been proposed for direct coupling, but there is no single LC-MS interface for all samples or all LC conditions.[56]

LC-MS is an "odd coupling". First, due of the nature of the analytes; since LC-MS is preferred over gas chromatography because of the higher polarity and lower volatility of the samples and one of the prerequisites of MS analysis is the formation of volatilized ions. Second, and harder to solve, is the necessary elimination of the mobile phase, because MS functions in gaseous phase. Beyond that, a flow-rate of 1 mL min^{-1} is far too much to be handled by standard MS vacuum systems. Therefore, to obtain a lower flow, we can use a flow splitter at the exit of the column (in case of analytical columns ID 0.46cm), or a straighter column (ID 0.21cm), which can work at lower flows (0.2 mL min^{-1}).[57]

1.6 References

[1] S.S. Nielsen; *Food Analysis*; 3rd edition. New York; Springer New York; 2003.

[2] P.C. Dinis, P.L. Falé, P.J.A. Madeira, M.H. Florêncio, M.L. Serralheiro; *Acetylcholinesterase Inhibitory Activity After in vitro Gastrointestinal Digestion of Infusions of Mentha Species*. European Journal of Medicinal Plants. 3 (2003) 381-93.

[3] M. Abdel-Mogib, H. Albar, S. Batterjee; *Chemistry of the Genus Plectranthus*. Molecules. 7 (2002) 271-301.

[4] C.W. Lukhoba, M.S.J. Simmonds, A.J. Paton; *Plectranthus: A review of ethnobotanical uses*. Journal of Ethnopharmacology. 103 (2006) 1-24.

[5] S. Waldia, B.C. Joshi, U. Pathak, M.C. Joshi; *The Genus Plectranthus in India and Its Chemistry*. Chemistry & Biodiversity. 8 (2011) 244-52.

[6] R. Kubínová, E. Švajdlenka, K. Schneiderová, Z. Hanáková, S. Dall'Acqua, O. Farsa; *Polyphenols and diterpenoids from Plectranthus forsteri 'Marginatus'*. Biochemical Systematics and Ecology. 49 (2013) 39-42.

[7] M. Petersen, M.S.J. Simmonds; *Rosmarinic acid*. Phytochemistry. 62 (2003) 121-5.

[8] N. Fabre, I. Rustan, E. de Hoffmann, J. Quetin-Leclercq; *Determination of flavone, flavonol, and flavanone aglycones by negative ion liquid chromatography electrospray ion trap mass spectrometry*. Journal of the American Society for Mass Spectrometry. 12 (2001) 707-15.

[9] F. Cuyckens, M. Claeys; *Mass spectrometry in the structural analysis of flavonoids*. Journal of Mass Spectrometry. 39 (2004) 1-15.

[10] K.B. Pandey, S.I. Rizvi; *Plant polyphenols as dietary antioxidants in human health and disease*. Oxidative Medicine and Cellular Longevity. 2 (2009) 270-8.

[11] R.E. March, E.G. Lewars, C.J. Stadey, X.-S. Miao, X. Zhao, C.D. Metcalfe; *A comparison of flavonoid glycosides by electrospray tandem mass spectrometry*. International Journal of Mass Spectrometry. 248 (2006) 61-85.

[12] Y.L. Ma, Q.M. Li, H. Van den Heuvel, M. Claeys; *Characterization of flavone and flavonol aglycones by collision-induced dissociation tandem mass spectrometry*. Rapid Communications in Mass Spectrometry. 11 (1997) 1357-64.

[13] A. Wojakowska, A. Piasecka, P.M. García-López, F. Zamora-Natera, P. Krajewski, Ł. Marczak, et al.; *Structural analysis and profiling of phenolic secondary metabolites of Mexican lupine species using LC–MS techniques*. Phytochemistry. 92 (2013) 71-86.

[14] P.J.A. Madeira, C.M. Borges, M.H. Florêncio; *Electrospray ionization Fourier transform ion cyclotron resonance mass spectrometric and semi-empirical calculations study of five isoflavone aglycones*. Rapid Communications in Mass Spectrometry. 24 (2010) 3432-40.

[15] J. Zhang, M.B. Satterfield, J.S. Brodbelt, S.J. Britz, B. Clevidence, J.A. Novotny; *Structural Characterization and Detection of Kale Flavonoids by Electrospray Ionization Mass Spectrometry*. Analytical Chemistry. 75 (2003) 6401-7.

[16] P.S.H.W.a.R.G. Cooks; *Ion Trap Mass Spectrometry*. Current Separations and Drug Development. 16 (1997) 1-8.

[17] P.J.A. Madeira, M.H. Florêncio; Applications of Tandem Mass Spectrometry: From Structural Analysis to Fundamental Studies. In: *Tandem Mass Spectrometry - Applications and Principles*; 1st edition. Croatia; InTech; 2012. p. 3-32.

[18] J. Barker; *Mass Spectrometry*; 2nd edition. Chichester; Wiley & Sons; 1999.

[19] G. Zeng, H. Xiao, J. Liu, X. Liang; *Identification of phenolic constituents in Radix Salvia miltiorrhizae by liquid chromatography/electrospray ionization mass spectrometry*. Rapid Communications in Mass Spectrometry. 20 (2006) 499-506.

[20] J. Griffiths; *A Brief History of Mass Spectrometry*. Analytical Chemistry. 80 (2008) 5678-83.

[21] J.J. Thomson; *Cathode Rays*. Philosophical Magazine Series. 5 44(269) (1897) 293-316.

[22] J.J. Thomson; *On Rays of Positive Electricity*. Philosophical Magazine Series. 6 13(77) (1907) 561-75.

[23] F.W. Aston; *A positive ray spectrograph*. Philosophical Magazine Series. 6 38(228) (1919) 707 - 14.

[24] A.J. Dempster; *A new Method of Positive Ray Analysis*. Physical Review. 11(4) (1918) 316-25.

[25] W. Bleakney; *A New Method of Positive Ray Analysis and Its Application to the Measurement of Ionization Potentials in Mercury Vapor*. Physical Review. 34(1) (1929) 157-60.

[26] H. Sommer, H.A. Thomas, e. al.; *The Measurement of e/M by Cyclotron Resonance*. Physical Review. 82(5) (1951) 697-702.

[27] M.M. Wolff, W.E. Stephens; *A Pulsed Mass Spectrometer with Time Dispersion*. Review of Scientific Instruments. 24(8) (1953) 616-7.

[28] W. Paul, H. Steinwedel; *A New Mass Spectrometer without a Magnetic Field*. Zeitschrift für Naturforschung A. 8 (7) (1953) 448–50.

[29] K.R. Jennings; *Collision-induced decompositions of aromatic molecular ions*. International Journal of Mass Spectrometry and Ion Physics. 1(3) (1968) 227-35.

[30] M.S.B. Munson, F.H. Field; *Chemical Ionization Mass Spectrometry. I. General Introduction*. Journal of the American Chemical Society. 88(12) (1966) 2621-30.

[31] M. Barber, R.S. Bordoli, e. al.; *Fast atom bombardment of solids (F.A.B.): a new ion source for mass spectrometry*. Journal of the Chemical Society, Chemical Communications. 7 (1981) 325-7.

[32] M. Karas, F. Hillenkamp; *Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons*. Analytical Chemistry. 60(20) (2002) 2299-301.

[33] J.B. Fenn, M. Mann, e. al.; *Electrospray ionization for mass spectrometry of large biomolecules*. Science. 246(4926) (1989) 64-71.

[34] E.d. Hoffmann, V. Stroobant; *Mass Spectrometry Principles and Applications*; 3rd edition. West Sussex; Wiley & Sons; 2007.

[35] T.F.P. Jorge; *Nutrientes e suplementos nutricionais em vegetais e frutos frequentemente usados na dieta: estudo por espectrometria de massa do resveratrol nas uvas*. Lisboa; Universidade de Lisboa; 2012.

[36] A. Makarov; *Electrostatic Axially Harmonic Orbital Trapping: A High-Performance Technique of Mass Analysis*. Analytical Chemistry. 72 (2000) 1156-62.

[37] J.W. Hager; *A new linear ion trap mass spectrometer*. Rapid Communications in Mass Spectrometry. 16 (2002) 512-26.

[38] C. Flender, C. Wolf, P. Leonhard, M. Karas; *Nano-liquid chromatography—direct electron ionization mass spectrometry: improving performance by a new ion source adapter*. Journal of Mass Spectrometry. 46 (2011) 1004-10.

[39] P.J.A. Madeira; *A mass spectrometry study of compounds with environmental and biological interest*. Lisboa; Universidade de Lisboa; 2010.

[40] S.E.V. Bramer; *An Introduction to Mass Spectrometry*; Chester; Widener; 1998.

[41] M. Dole, L.L. Mack, R.L. Hines, R.C. Mobley, L.D. Ferguson, M.B. Alice; *Molecular Beams of Macroions*. Journal of Chemical Physics. 49 (5) (1968) 2240-9.

[42] S.J. Gaskell; *Electrospray: Principles and Practice*. Journal of Mass Spectrometry. 32 (1997) 677-88.

[43] N.B. Cech, C.G. Enke; *Practical implications of some recent studies in electrospray ionization fundamentals*. Mass Spectrometry Reviews. 20 (2001) 362-87.

[44] L. Konermann, E. Ahadi, A.D. Rodriguez, S. Vahidi; *Unraveling the Mechanism of Electrospray Ionization*. Analytical Chemistry. 85 (2012) 2-9.

[45] D.C. Harris; *Quantitative Chemical Analysis*; 8th edition. New York; Clancy Marshall; 2010.

[46] J. Irbarne, B. Thomson; *On the evaporation of small ions from charged droplets*. Nature Biotechnology. 64 (1976) 2287-94.

[47] A.R.R. Gomes; *Correlação da função antioxidante com a energia de ligação O-H em compostos fenólicos.* Lisboa; Universidade de Lisboa; 2012.

[48] W. Paul, H. Steinwedel; *Apparatus for Separating Charged Particles of Different Specific Charges*. 1956: German Patent.

[49] W. Paul, H. Steinwedel; *Apparatus for separating charged particles of different specific charges*. 1960: US Patent.

[50] G.C. Stafford, P.E. Kelley, J.E.P. Syka, W.E. Reynolds, J.F.J. Todd; *Recent Improvements In and Analytical Applications of Advanced Ion Trap Technology*. International Journal of Mass Spectrometry and Ion Processes. 60 (1984) 85-98.

[51] A.K. Shukla, J.H. Futrell; *Tandem mass spectrometry: dissociation of ions by collisional activation*. Journal of Mass Spectrometry. 35 (2000) 1069-90.

[52] K.R. Jennings; *Analytical applications of ion activation techniques*. International Journal of Mass Spectrometry. (2014) http://dx.doi.org/10.1016/j.ijms.2014.05.017.

[53] M. Tswett; *Physical-chemical studies of chlorophyll. Adsorption*. Berichte der Deutschen botanischen Gesellschaft. 24 (1906) 316-26.

[54] http://www.medicinal-chemistry.org/files/harki/TTL%20Presentation_Jan2013.pdf; 26 February 2014.

[55] http://www.waters.com/waters/pt_PT/HPLC---High-Performance-Liquid-Chromatography/nav.htm?cid=10048919&locale=pt_PT; 26 February 2014.

[56] L.R. Snyder, J.J. Kirkland, J.W. Dolan; *Introduction to Modern Liquid Chromatography*; 3rd edition. New Jersey; Wiley & Sons; 2010.

[57] F. Lemière; *Interfaces for LC–MS*; Guide to LC–MS 2001.

CHAPTER 2

EXPERIMENTAL SECTION

2.1. Materials

All weighings were performed using a Sartorius CP225D analytical balance from DWS (USA). During sample preparation, various materials were used beyond the general laboratory equipment: a Heto PowerDry LL3000 freeze dryer from ThermoFisher Scientific (USA); a vortex mixer from VELP Scientifica (Italy); a Galaxy 7D-230V-EU digital microcentrifuge from VWR (USA); Injekt 5 mL syringes from Braun (Germany); Sterican 0.80 x 50mm needles from Braun (Germany); 0.45µm syringe filters from Whatman (USA); Oasis HLB SPE cartridges 30 µm from Waters (USA).

2.2. Reagents

All of the reagents used during this work were of analytical grade and did not undergo any further purification.

The methanol (MeOH) and the formic acid (HCOOH) were purchased from Carlo Erba Reagents, France. The ammonium hydroxide (NH_4OH) solution and the sodium chloride (NaCl) were obtained from Sigma-Aldrich, Germany. The ethyl acetate (EtOAc) and the trifluoroacetic acid (TFA) were purchased from Merck, Germany. The ammonium acetate (NH_4OAc) was acquired from Fisher Chemical (UK). The water (H_2O) - resistivity 18.2MΩ.cm, 25°C - was purified using a Milli-Q water system from Millipore (USA). The helium gas used as bath gas in the ion trap was purchased from Praxair (Portugal).

2.3. Standard preparation

The five flavones under study (**Figure 2.1/Table 2.1**) were purchased from Extrasynthese, France. Two solutions of each flavone were prepared in HPLC grade MeOH at a concentration of 10^{-5} M. The solutions analysed in the positive ion mode were acidified with 2 µL of HCOOH.

To study the formation of adducts between these compounds and the ammonium ion (NH_4^+), a NH_4OAc solution was prepared at a concentration of 10^{-2}M. 1µL of this solution was added to each compound solution. The same was performed using NaCl, to study the adduct formation between the compounds and the sodium ion (Na^+).

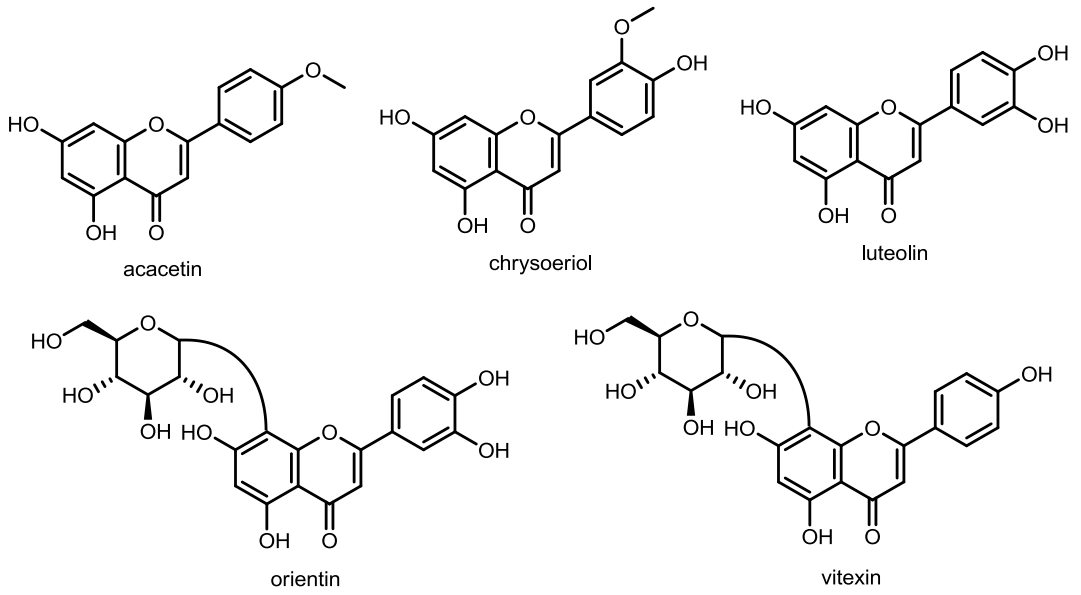


Figure 2.1 – Structures of the flavone standards under study.

Table 2.1 – Molecular masses of the flavones.

Flavone	Molecular mass (g mol^{-1})
Acacetin	284
Chrysoeriol	300
Luteolin	286
Orientin	448
Vitexin	432

2.4. MS^n analysis conditions

The MS^n experiments were performed with a LCQ Duo ion trap mass spectrometer from Thermo Scientific (USA) equipped with an ESI source. The standard solutions were introduced using a syringe pump, at a flow rate of 500 µL h^{-1}. The applied spray voltage in the source was 4.50 kV, the capillary voltage was 10 V and the capillary temperature 220°C. The sheath gas (nitrogen) flow was 40 a.u. and the auxiliary gas (nitrogen) flow was 20 a.u. The mass spectrometer parameters were adjusted to optimize the signal-to-noise ratios for the ions of interest. Helium was used as collision gas to perform the MS^n experiments. The ions of interest were activated and the collision energy was gradually increased. All mass spectrometry data were acquired in the positive and negative ion modes, using Xcalibur version 1.2 from Thermo Scientific (USA).

2.5. Semi-empirical calculations

To predict the protonation and deprotonation sites of the five flavones, semi-empirical calculations were performed using PM6 hamiltonean [1] implemented in MOPAC2012.[2,3] Heats of formation ($\Delta_f H$) were calculated for the neutral and the various protonated and deprotonated forms of each compound, which were then used to calculate the reaction enthalpies ($\Delta_r H$), as shown in the equations below.

Equation 2.1 $\mathbf{M + H^+} \longrightarrow \mathbf{MH^+}$ (protonation)

Equation 2.2 $\mathbf{\Delta_r H}$ (prot) $= \mathbf{\Delta_f H\ (MH^+) - [\Delta_f H\ (M) + \Delta_f H\ (H^+)]}$

Equation 2.3 $\mathbf{MH} \longrightarrow \mathbf{M^- + H^+}$ (deprotonation)

Equation 2.4 $\mathbf{\Delta_r H}$ (deprot) $= \mathbf{[\Delta_f H\ (M) + \Delta_f H\ (H^+)] - \Delta_f H\ (MH)}$

For the calculation of these values it was assumed that $\Delta_f H\ (H^+) = 1530$ kJ mol^{-1}, according to the so-called Ion Convention.[4]

Frequency calculations were performed using the force keyword to determine if the calculated energy corresponds to a real energy minimum or if it is just a local minimum.
The software Avogadro was used to visualize the MOPAC2012 outputs.[5]

2.6. Sample preparation

The leaves of ten different *Plectranthus* species (**Table 2.2**) were boiled in water for about 10 minutes. Then the water was filtered, frozen and lyophilized (-52°C). 10 mg of each extract were weighed and dissolved in 1 mL of MeOH and H_2O (50/50). The solutions were agitated in a vortex mixer for a few seconds and after centrifuged for 5 minutes. The supernatant was filtered through a syringe filter (pore size 0.45 μm) into a vial.

Table 2.2 – The ten *Plectranthus* species analysed in this work.

***Plectranthus* species**	
***P. gradientatus* (PG)**	***P. zuluensis* (PZ)**
***P. lanuginosus* (PL)**	***P. verticilatus* (PV)**
***P. verticilatus u. bombo* (PVUB)**	***P. ersti* (PE)**
***P. neochilus* (PN)**	***P. ventrii* (PVEN)**
***P. apimentado* (PA)**	***P. madagoscariensis* (PM)**

2.6.1. Solid phase extraction (SPE)

The Oasis HLB cartridge was conditioned with 2 x 3 mL of H_2O, 2 x 3 mL of MeOH and 2 x 3 mL of EtOAc. The samples were then eluted in three fractions: 2 x 2 mL of H_2O, 2 x 2 mL of MeOH and 2 x 2 mL of EtOAc. After the evaporation of the solvent, the mobile phase reconstituted with MeOH and H_2O (50/50) and injected in the LC-MS system.

2.7. LC-MS^n analysis conditions

The LC-MS^n analyses were performed with a HPLC Surveyor Plus from Thermo Scientific (USA) coupled to a LCQ Duo ion trap mass spectrometer from Thermo Scientific (USA) equipped with an ESI source. The chromatographic column that was used was a LiChrospher 100 RP-8 (5µm) LiChroCART 250-4 mm from Merck (Germany).

Five different methods were tested, differing between them the eluent constitution, the flow rate and the time of the runs. The injection volume for the five methods was 25 µL. When the mobile phase flow was 1 mL min^{-1} a flow splitter was used at the exit of the column.

Method A

Method A consisted in 30 min runs and a mobile phase constituted by a binary system of MeOH (A) and H_2O + 0.05% TFA (B), at a flow rate of 1 mL min^{-1}.

Table 2.3 – HPLC gradient program used for method A.

Time (min)	% A	% B	Flow (mL min^{-1})
0	20	80	1
20	80	20	1
25	80	20	1
28	20	80	1
30	20	80	1

Method B

Method B consisted in 30 min runs and a mobile phase constituted by a binary system of MeOH (A) and H_2O + 1% HCOOH (B), at a flow rate of 1 mL min^{-1}.

Table 2.4 – HPLC gradient program used for method B.

Time (min)	% A	% B	Flow (mL min^{-1})
0	30	70	1
20	80	20	1
25	80	20	1
28	30	70	1
30	30	70	1

Method C

Method C consisted in 30 min runs and a mobile phase constituted by a binary system of MeOH (A) and H_2O + 0.1% HCOOH (B), at a flow rate of 1 mL min^{-1}.

Table 2.5 – HPLC gradient program used for method C.

Time (min)	% A	% B	Flow (mL min^{-1})
0	30	70	1
20	80	20	1
25	80	20	1
28	30	70	1
30	30	70	1

Method D

Method D consisted in 30 min runs and a mobile phase constituted by a binary system of MeOH (A) and H_2O + 0.1% NH_4OH solution (B), at a flow rate of 1 mL min^{-1}.

Table 2.6 – HPLC gradient program used for method D.

Time (min)	% A	% B	Flow (mL min^{-1})
0	30	70	1
20	80	20	1
25	80	20	1
28	30	70	1
30	30	70	1

Method E

Method E consisted in 90 min runs and a mobile phase constituted by a binary system of MeOH (A) and H_2O + 0.1% HCOOH (B), at a flow rate of 0.3 mL min^{-1}.

Table 2.7 – HPLC gradient program used for method E.

Time (min)	% A	% B	Flow (mL min^{-1})
0	30	70	0.3
45	80	20	0.3
46	99	1	0.3
60	99	1	0.3
61	30	70	0.3
90	30	70	0.3

The mass spectrometer parameters were adjusted to optimize the signal-to-noise ratios. The applied spray voltage in the source was 4.50 kV and the capillary temperature 220°C. The sheath gas (nitrogen) flow was 40 a.u. and the auxiliary gas (nitrogen) flow was 20 a.u. Helium was used as collision gas. The MS^n experiments were accomplished by isolating the ions of interest in the ion trap and colliding them with the collision gas. All experiments were performed in the positive and negative ion modes. The MS spectra were acquired and processed by Xcalibur version 1.2 from Thermo Scientific (USA).

2.8. References

[1] J.P. Stewart; *Optimization of parameters for semiempirical methods V: Modification of NDDO approximations and application to 70 elements*. Journal of Molecular Modelling. 13 (2007) 1173-213.

[2] MOPAC2012, J.J.P. Stewart, Stewart Computational Chemistry, Version 13.326L http://OpenMOPAC.net; 16 July 2014.

[3] J.D.C. Maia; Journal of Chemical Theory and Computation. 8 (2012) 3072-3081.

[4] S.G. Lias, J.E. Bartmess; *Gas-phase ion thermochemistry*. http://webbook.nist.gov/chemistry/ion; 16 July 2014.

[5] Advogadro: an open-source molecular builder and visualization tool. Version 1.0.1. http://avogadro.openmolecules.net/; 16 July 2014.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Study of the gas-phase behaviour of flavones

3.1.1. Protonation and deprotonation sites by semi-empirical calculations

Five flavones (acacetin, chrysoeriol, luteolin, orientin and vitexin) were studied by electrospray ionization mass spectrometry in the positive and negative ion modes.

First of all we needed to determine the most probable protonation and deprotonation sites for each compound, by computing the ΔH for the protonation and deprotonation reactions using semi-empirical calculations as described in chapter 2. The deprotonation can occur on any of the hydroxyl (OH) groups, while the protonation can occur on any group containing an oxygen atom. The general structures and the numbering schemes are presented in **Figure 3.1**. The protonation and deprotonation reaction values ΔrH (kJ mol^{-1}) are presented in **Table 3.1** and **Table 3.2**, respectively.

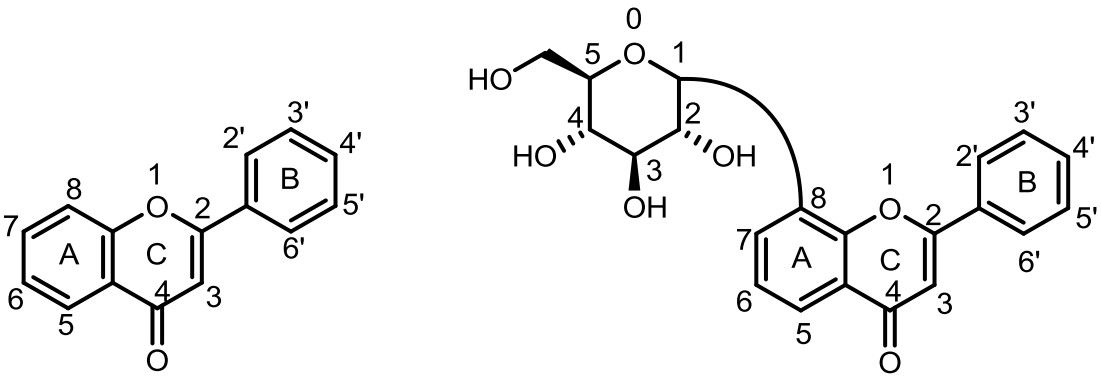


Figure 3.1 – General flavone and flavone-8-*C*-glucoside structures and their respective numbering scheme.

Table 3.1 – Δ_rH values (kJ mol^{-1}) for the different protonation sites of the five flavones.

Protonation site	Acacetin	Chrysoeriol	Luteolin	Orientin	Vitexin
1	1940.663	-726.571	-719.511	-816.534	-811.756
4	-898.154	-885.879	-880.759	-878.028	-879.733
5	-906.802	-896.779	-891.819	-888.793	-888.596
7	-734.935	-730.521	-726.869	-823.546	-820.943
4'	-741.949	-756.120	-743.741	-765.988	-733.461
5'	-	-755.782	-745.170	-763.753	-
0 (sugar)	-	-	-	-863.072	-859.655
2 (sugar)	-	-	-	-823.545	-820.943

Table 3.1 – $\Delta_r H$ values (kJ mol^{-1}) for the different protonation sites of the five flavones (continuation).

3 (sugar)	-	-	-	-835.601	-829.299
4 (sugar)	-	-	-	-861.805	-851.858
5 (sugar)	-	-	-	-873.943	-859.069

Table 3.2 – $\Delta_r H$ values (kJ mol^{-1}) for the different deprotonation sites of the five flavones.

Deprotonation site	Acacetin	Chrysoeriol	Luteolin	Orientin	Vitexin
5	1371.952	1364.721	1360.449	1323.094	1334.447
7	1316.052	1311.243	1307.241	1255.493	1266.539
4'	-	1293.320	1273.261	1258.514	1278.913
5'	-	-	1314.764	1242.909	-
2 (sugar)	-	-	-	1341.122	1266.539
3 (sugar)	-	-	-	1242.261	1251.402
4 (sugar)	-	-	-	1220.970	1231.407
5 (sugar)	-	-	-	1255.493	1348.878

The most probable protonation/deprotonation site corresponds to the one that presents the lowest energy form of them all. So, taking into account the semi-empirical calculations performed, the most probable protonation site is assumed to be at position 5 OH group for all flavones, since it is where $\Delta_r H$ is lower. However, as can be seen in **Figure 3.2**, the proton (H^+) migrated from position 5 OH group of ring A to position 4 C=O group of ring C. In **Table 3.1**, it can be seen that the $\Delta_r H$ values only differ about 10 kJ mol^{-1} between protonation positions 4 and 5. Therefore, we can assume that the protonation is more likely to occur at position 4 C=O group for the five flavones under study. This protonated structure could be more stable than the rest due to the formation of a hydrogen bond between the hydrogen atom at position 4 and the oxygen atom at position 5. These structures also indicate that there was charge relocation through ring C to the oxygen atom at position 1.

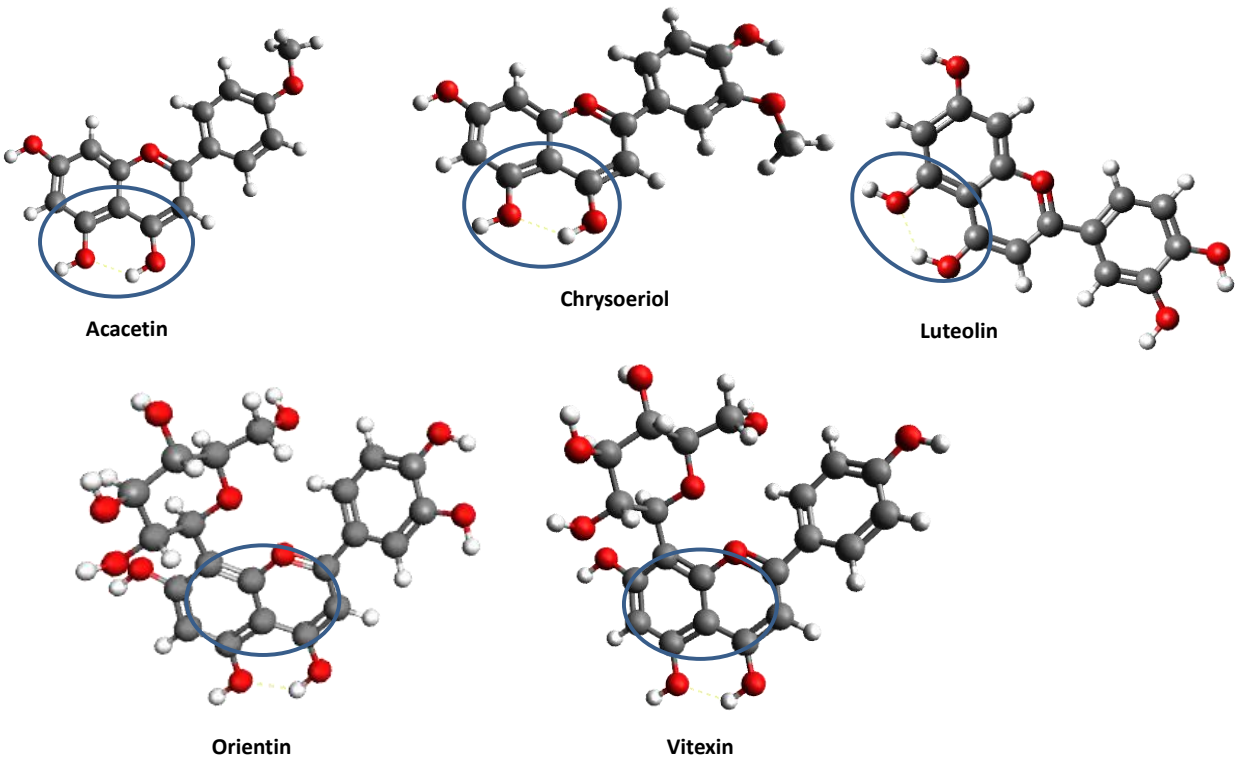


Figure 3.2 – Optimized structures of the five flavones protonated at position 4 C=O group instead of at position 5 OH group.

Deprotonation is predicted to occur at the 7 OH group for acacetin, at the 4' OH group for chrysoeriol and luteolin, and at the 4 (sugar) OH group for both orientin and vitexin (**Table 3.3**). Semi-empirical calculations have already proved to be accurate enough to resolve mass spectrometry problems.[1] Furthermore, density functional theory (DFT) calculations, performed to access the gas-phase acidity of flavonoids, also predicted that luteolin would deprotonate preferentially at the 4' OH group.[2]

Table 3.3 – Predicted protonation and deprotonation sites for each flavone under study.

Flavone	Protonation	Deprotonation
Acacetin	4	7
Chrysoeriol	4	4'
Luteolin	4	4'
Orientin	4	4 (sugar)
Vitexin	4	4 (sugar)

3.1.2. ESI-MS and ESI-MS^n of the flavones under study

A solution of each compound was injected in the mass spectrometer at a concentration of 10^{-5} M and MS^n spectra were acquired in the positive and negative ion modes, according to the conditions described in chapter 2.

The MS spectrum of each flavone in the negative ion mode is presented in **Figure 3.3**.

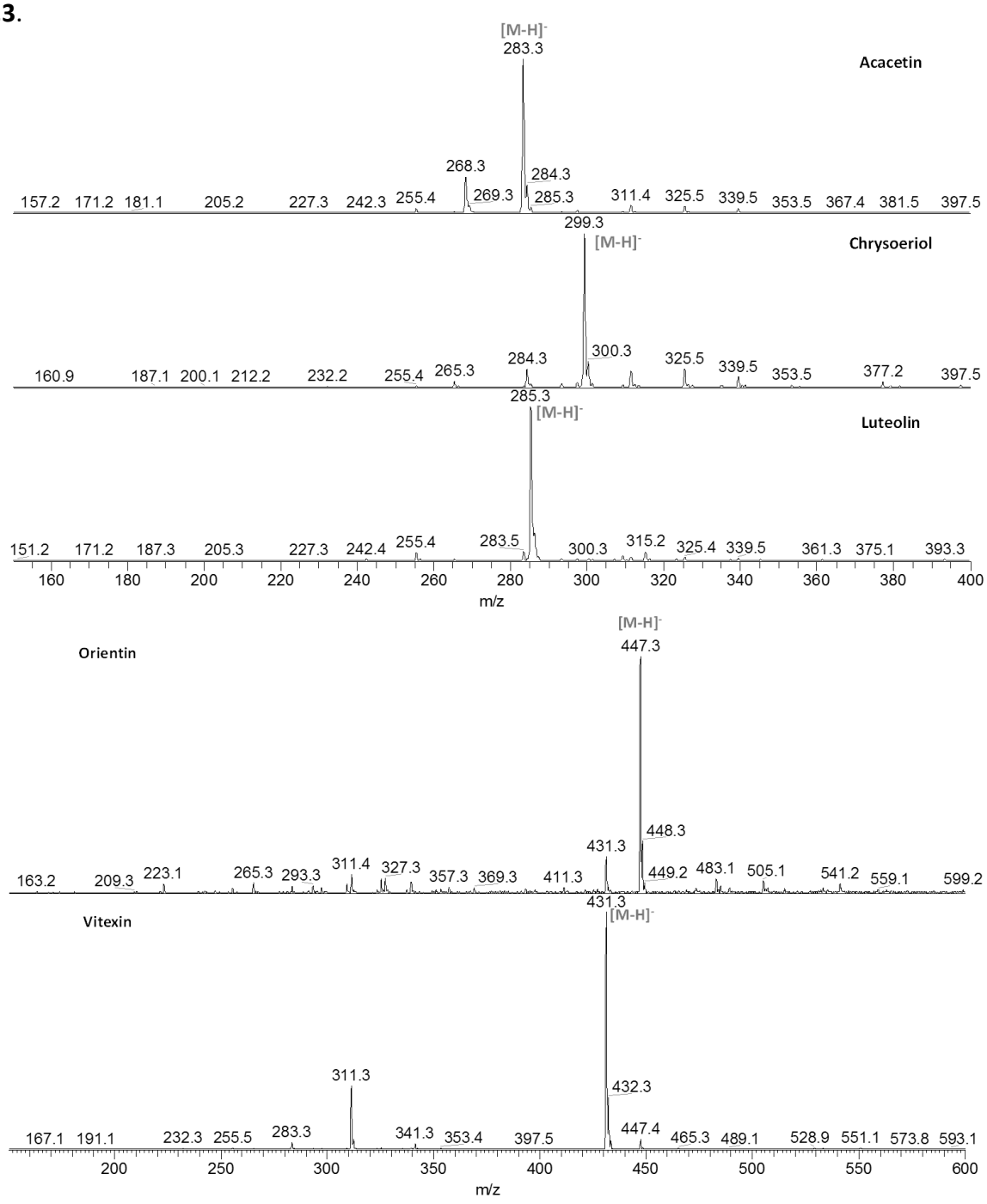


Figure 3.3 – Full MS spectrum of each flavone in the negative ion mode.

The product ions are identified according to the nomenclature developed by Claeys and co-workers[3], adapted from those developed by Mabry and Markham[4] and Domon and Costello[5].

Table 3.4 – Product ions corresponding to the mass losses observed for the five flavones in the negative ion mode.

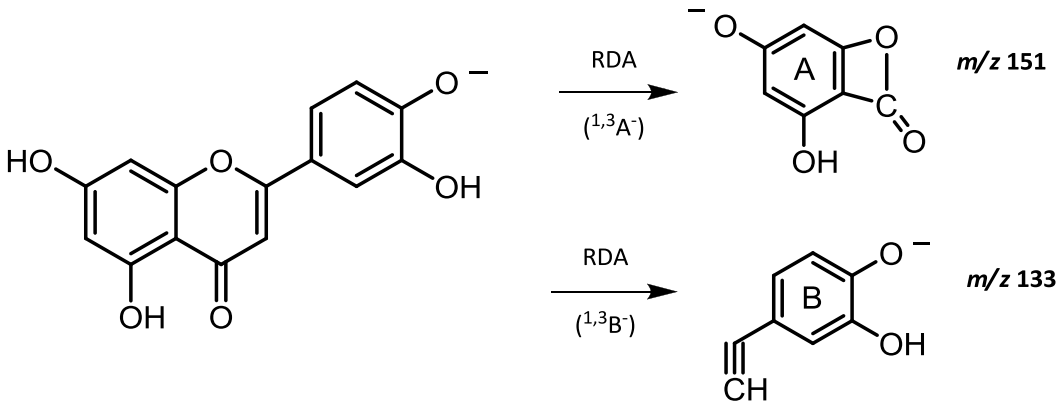
	Acacetin	Chrysoeriol	Luteolin	Orientin	Vitexin
$[M-H]^-$	283	299	285	447	431
$[M-H-H_2O]^-$	265	281	267	429	
$[M-H-2H_2O]^-$				411	
$[M-H-3H_2O]^-$				393	
$[M-H-CH_3]^{\cdot-}$	268	284			
$[M-H-CO]^-$	255	271	257		
$[M-H-CO_2]^-$	239	255	241		387
$[M-H-2CO_2]^-$	195	211	197		
$[M-H-CO_2-CO]^-$	212	227	213	375	
$[M-H-CH_3OH]^{\cdot-}$		267			
$[M-H-CH_3-CO]^{\cdot-}$	240	256	242		
$[M-H-C_2H_2O]^-$	241	257	243		
$[M-H-C_2H_2O-CO_2]^-$	197	213	199		
$[M-H-CO_2-CO-CH_3]^{\cdot-}$			198		
$[M-H-CO_2-H_2O]^-$			223		
$[M-H-C_3O_2]^-$		231	217		
$[M-H-C_3O_2-CH_3]^{\cdot-}$	200				
$[M-H-C_3O_2-C_2H_2O]^-$			175		
$[M-H-C_3O_2-CH_3-CO]^{\cdot-}$	172	188			
$[M-H-CH_2O-H_2O]^-$					383
$^{1,3}A^-$ (RDA)	151	151	151		
$^{1,3}B^-$ (RDA)	131	147	133	133	
$^{0,4}B^-$	175		177		
$^{0,4}B^- - H_2O$	157		159		
$^{0,2}B^-$	133		135		
$^{0,1}X^-$				297	
$^{0,1}X^- - H_2O$				279	
$^{0,1}X^- - CO$				269	
$^{0,1}X^- - CO_2$				253	
$^{0,1}X^- - CO_2 - CO$				225	
$^{0,1}X^- - H_2O - CO$				251	
$^{0,1}X^- - C_2H_2O$				255	
$^{0,1}X^- - C_2H_2O - CO_2$				211	
$^{0,2}X^-$				327	311
$^{0,2}X^- - H_2O$				309	
$^{0,2}X^- - CO$				299	283
$^{0,2}X^- - 2CO$				271	255
$^{0,2}X^- - CO_2 - CO$					239
$^{0,2}X^- - CO_2 - CO - C_2H_2O$				213	

Table 3.4 – Product ions corresponding to the mass losses observed for the five flavones in the negative ion mode (continuation).

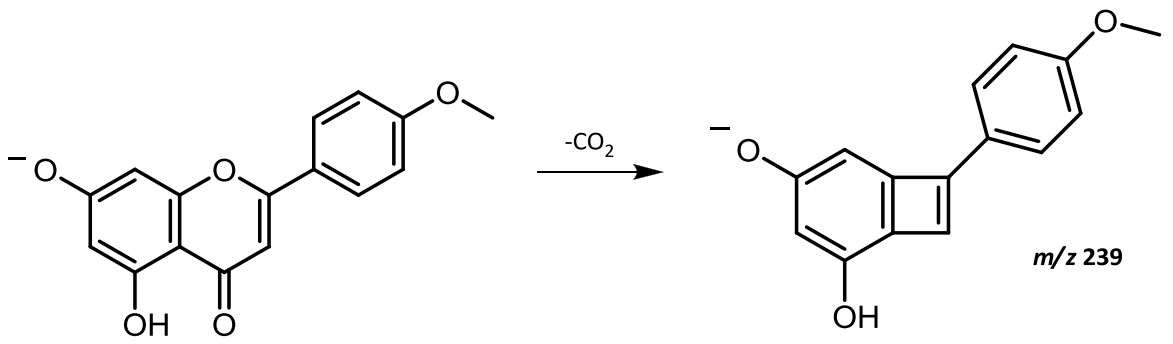
$^{0,2}X^-$-C_3O_2					243
$^{0,2}X^-$-C_2H_2O-CO_2					225
$^{0,2}X^-$-2H$^\cdot$					309
$^{0,3}X^-$				357	341
$^{0,3}X^-$-H_2O				339	323
$^{0,3}X^-$-H_2O-CH_3				324	
$^{0,3}X^-$-H_2O-CO				311	295
$^{0,3}X^-$-H_2O-CO_2				295	
$^{0,3}X^-$-H_2O-C_3O_2-C_2H_2O				229	
$^{0,4/1,3/2,4}X^-$-H_2O				369	
Y^-				285	269
Y^--H_2O				267	
Y^--CO_2				241	
Y^--C_3O_2				217	
Y^--C_3O_2-C_2H_2O				175	
Y^--H$^\cdot$				284	268

For the flavones under study, losses of H_2O, CO, CO_2, CH_3, C_2H_2O were identified, together with cross-ring cleavages of the aglycone and the sugar moiety, when applicable.

Cleavage of ring C by RDA mechanisms was identified for the three flavone aglycones, leading to $^{1,3}A^-$ and $^{1,3}B^-$ product ions. These fragmentations can be seen for luteolin, as example, in **Scheme 3.1**. The losses of CO and CO_2 can be attributed to ring C, which undergoes contraction. The loss of CO_2 can be seen for acacetin, as example, in **Scheme 3.2**. Chrysoeriol showed also a loss of 15 u resulting in a $[M\text{-}H\text{-}CH_3]^{-\cdot}$ radical ion, which is characteristic of methylated compounds.

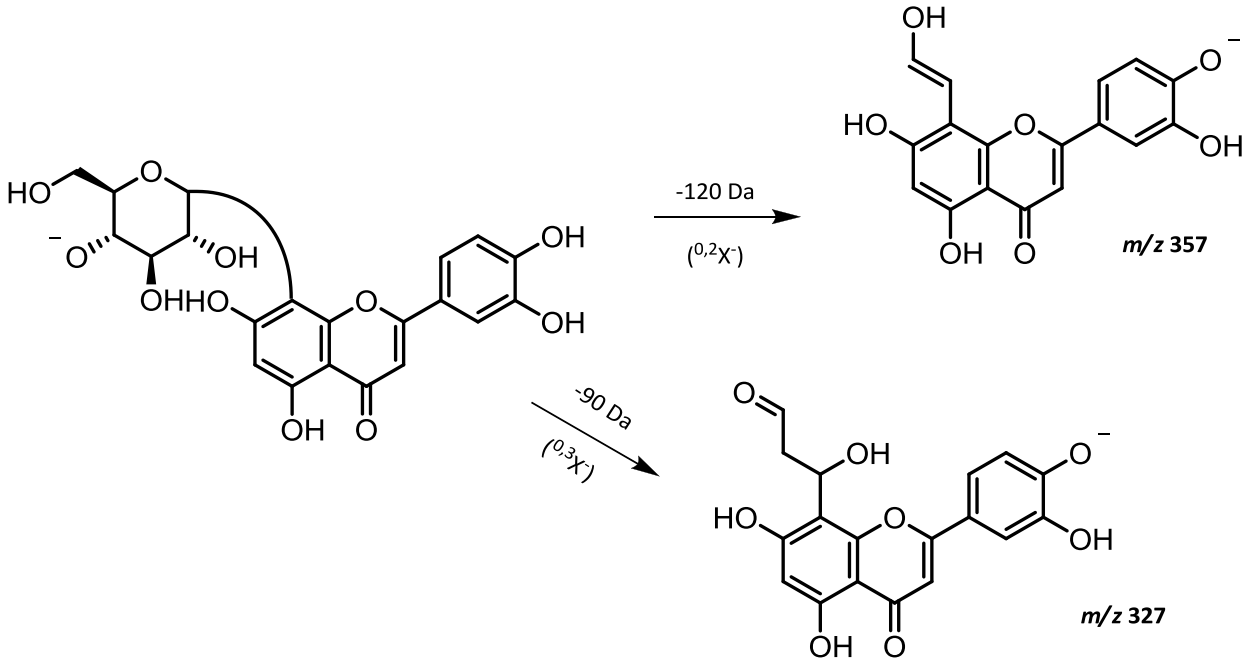


Scheme 3.1 – RDA fragmentation observed for luteolin, resulting in $^{1,3}A^-$ and $^{1,3}B^-$ product ions.



Scheme 3.2 – Loss of CO_2 observed for acacetin, resulting in contraction of ring C.

For the *C*-glycoside flavones, the major product ions are $^{0,2}X^-$ and $^{0,3}X^-$, corresponding to the losses of 90 and 120 Da, respectively. These cross-ring cleavages of the sugar moiety are characteristic diagnostic ions for *C*-glycoside flavones. Both product ions for orientin are shown in **Scheme 3.3**. For vitexin, the only difference remains in the functional group at position 5' (orientin: OH; vitexin: H).



Scheme 3.3 – Losses of 90 and 120 Da observed for orientin, corresponding to $^{0,3}X^-$ and $^{0,2}X^-$ ions, respectively.

The MS spectra of the flavones in the positive ion mode are presented in **Figure 3.4**.

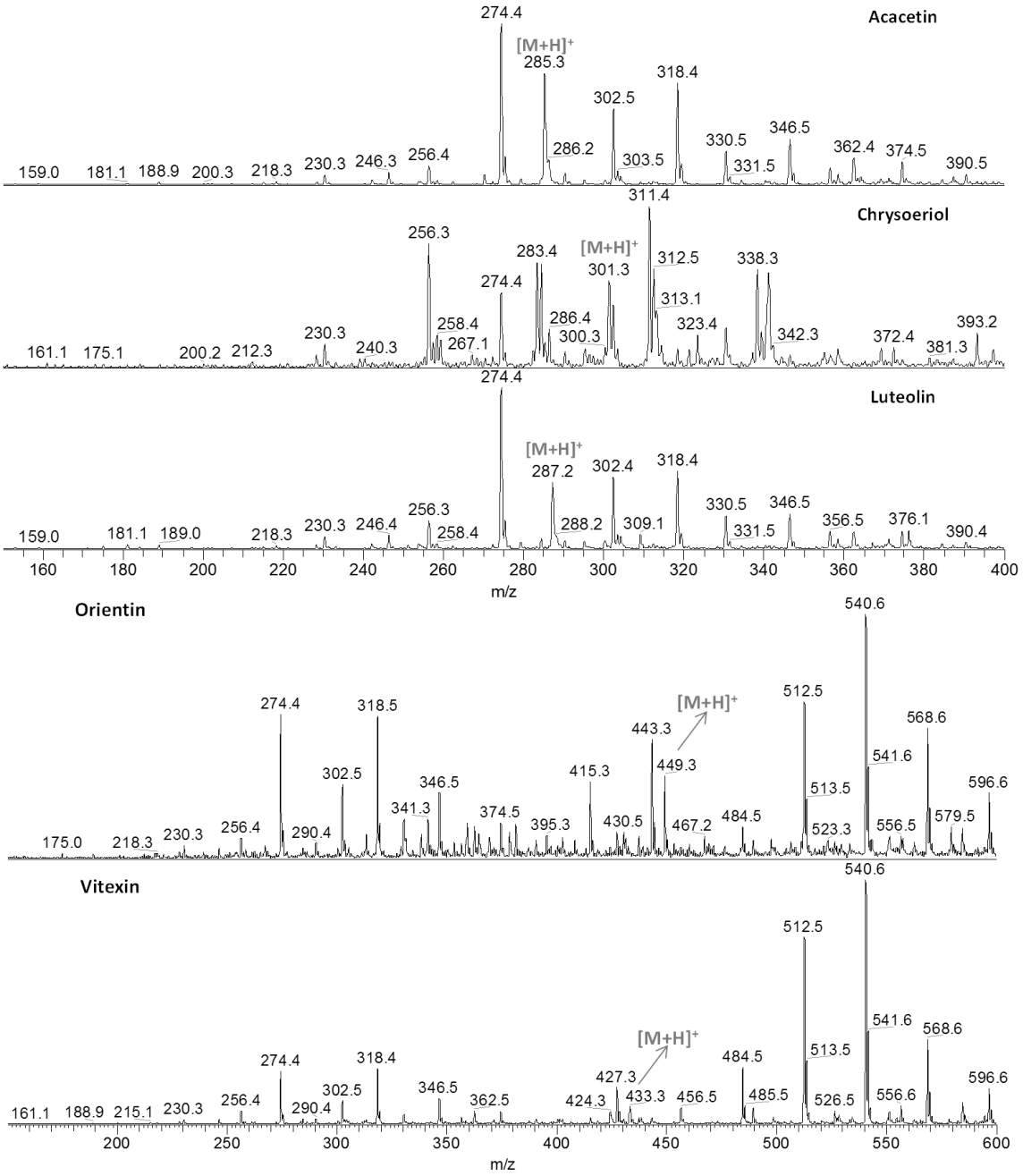


Figure 3.4 – Full MS spectrum of each flavone in the positive ion mode.

As for the negative ion mode product ions, the product ions in the positive ion mode are also identified according to the nomenclature developed by Claeys and co-workers[3], adapted from those developed by Mabry and Markham[4] and Domon and Costello[5].

Table 3.5 – Product ions corresponding to the mass losses observed for the five flavones in the positive ion mode.

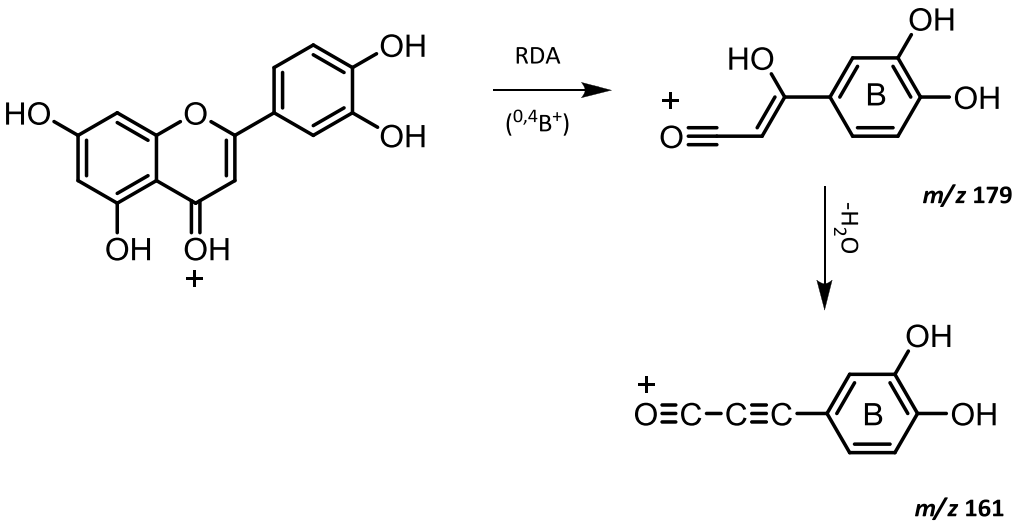
	Acacetin	Chrysoeriol	Luteolin	Orientin	Vitexin
$[M+H]^+$	285	301	287	449	433
$[M+H-H_2O]^+$	267	283	269	431	415
$[M+H-2H_2O]^+$				413	397
$[M+H-3H_2O]^+$				395	379
$[M+H-CH_3]^{+\cdot}$	270	286			418
$[M+H-CO]^+$	257	273	259		
$[M+H-H_2O-CO]^+$	239	255	241		
$[M+H-H_2O-K^+]^+$			230		
$[M+H-2H_2O-K^+]^+$			212		
$[M+H-3H_2O-C]^+$				383	
$[M+H-3H_2O-CO]^+$				367	351
$[M+H-3H_2O-2CO]^+$				339	
$[M+H-CH_3OH]^{+\cdot}$	253	269	255		
$[M+H-CH_3-CO]^{+\cdot}$	242	258	244		
$[M+H-C_2H_2O]^+$	243	259	245		
$[M+H-C_2H_2O-C_2H_2]^+$	217	233	219		
$[M+H-C_2H_2O-C_2H_2O]^+$	201	217	203		
$[M+H-C_2H_2O-C_2H_2-CH_3]^{+\cdot}$	202	218	204		
$[M+H-CH_3-CO-H_2O]^{+\cdot}$		240			
$[M+H-C_3O_2-H_2O]^+$		218			
$[M+H-19]^+$		282	268		
$[M+H-29]^+$			258		
$[M+H-183]^+$	102				
$[M+H-197]^+$	88				
$[M+H-105]^+$			182		
$^{1,3}A^+$ (RDA)	153	153	153		
$^{1,3}B^+$ (RDA)	133		135		
$^{0,4}B^+$	177	193	179	165	165
$^{0,4}B^+-H_2O$	159	175	161		
$^{0,2}B^+$	135	151	137	137	
$^{0,1}X^+$				299	283
$^{0,2}X^+$				329	313
$^{0,2}X^+-H_2O$				311	295
$^{0,4}X^+-2H_2O$					337
$^{0,4}X^+-2H_2O-CO$					309
$^{0,4/1,3/2,4}X^+-2H_2O$				353	
$^{1,2}X^+-2H_2O$					367
$^{1,2}X^+-4H_2O$					361
$^{1,5}X^+$					299

Table 3.5 – Product ions corresponding to the mass losses observed for the five flavones in the positive ion mode (continuation).

Y^+				287	271
Y				286	
Y^+-CO					243
Y^+-3H_2O					217

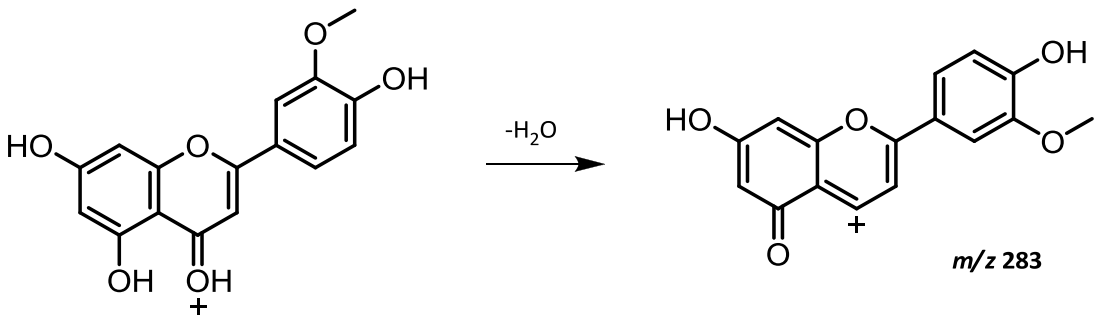
In the positive ion mode, for all flavones under study, losses of H_2O, CO, C_2H_2O were identified, together with cross-ring cleavages of the aglycone and the sugar moiety, when applicable.

Cleavage of ring C by RDA mechanisms was identified for the three flavone aglycones, leading to $^{1,3}A^+$ and $^{1,3}B^+$ product ions. An alternative RDA fragmentation pathway leads to $^{0,4}B^+$ product ions, which further fragment by the loss of a molecule of H_2O, yielding ($^{0,4}B^+$-H_2O) ions. This fragmentation type can be seen for luteolin, as example, in **Scheme 3.4**.



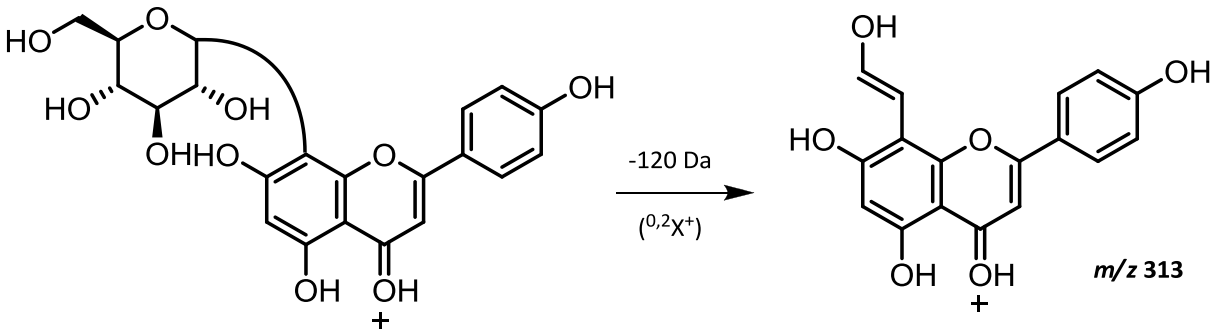
Scheme 3.4 – $^{0,4}B^+$ and $^{0,4}B^+$-H_2O product ion observed for luteolin.

The loss of H_2O could be induced by the protonation at position 4 group of ring C. An example of this loss can be seen for chrysoeriol in **Scheme 3.5**.



Scheme 3.5 – Loss of H_2O observed for chrysoeriol.

Although for the *C*-glycoside flavones the major product ions derive from the successive losses of molecules of H_2O, cross-ring cleavages of the sugar moiety, such as $^{0,1}X^+$ and $^{0,2}X^+$, can also be observed. In **Scheme 3.6**, $^{0,2}X^+$ product ion can be seen for vitexin, corresponding to a loss of 120 Da.



Scheme 3.6 – Loss of 120 Da observed for vitexin, corresponding to $^{0,2}X^+$ product ions.

The most useful fragmentations in the case of flavonoids are those that require the cleavage of two C-C bonds of ring C, which result in structurally informative ions ($^{I,j}A^{+/-}$ and $^{I,j}B^{+/-}$). These ions, which can be formed by retro-Diels-Alder (RDA) reactions, are the best diagnostic fragments for flavonoids as they provide information on the number and type of substituents in rings A and B.[6]

Although the negative ion mode seems to be more selective than the positive ion mode, the analysed results suggest that both ion modes are complementary tools for the structural characterization of flavones by mass spectrometry.

3.2. Analytical methodology

During this work various methods were used for the separation and identification of phenolic compounds and diterpenoids in *Plectranthus* extracts. A LiChrospher 100 RP-8 (5µm) LiChroCART 250-4 mm chromatographic column was used in all the experiments. All the HPLC methods started with a higher percentage of the aqueous phase, and at the end of each method the initial conditions were restored. The optimization of the chromatographic conditions includes the constitution of the eluent, the flow rate and the time of the runs.

The initial method (using as aqueous phase H_2O + TFA 0.05%) was an already developed method by members of the group for the analysis of other *Plectranthus* species. As can be seen in **Figure 3.5**, the chromatogram obtained with this method presents good peak resolution and various compounds were separated, as is expected when analysing such a complex matrix. However, as shown in **Figure 3.6**, the compounds formed adducts with the TFA, which complicated their identification. Therefore, to try to avoid adduct formation we used as aqueous phase H_2O + HCOOH 1% (in method B) and H_2O + HCOOH 0.1% (in method C). Both methods did not completely resolve the adduct problem, since some of the compounds formed adducts with the HCOOH. However, these methods produced more enlightening product ions, so their results could be used to identify the compounds present in the *Plectranthus* extracts.

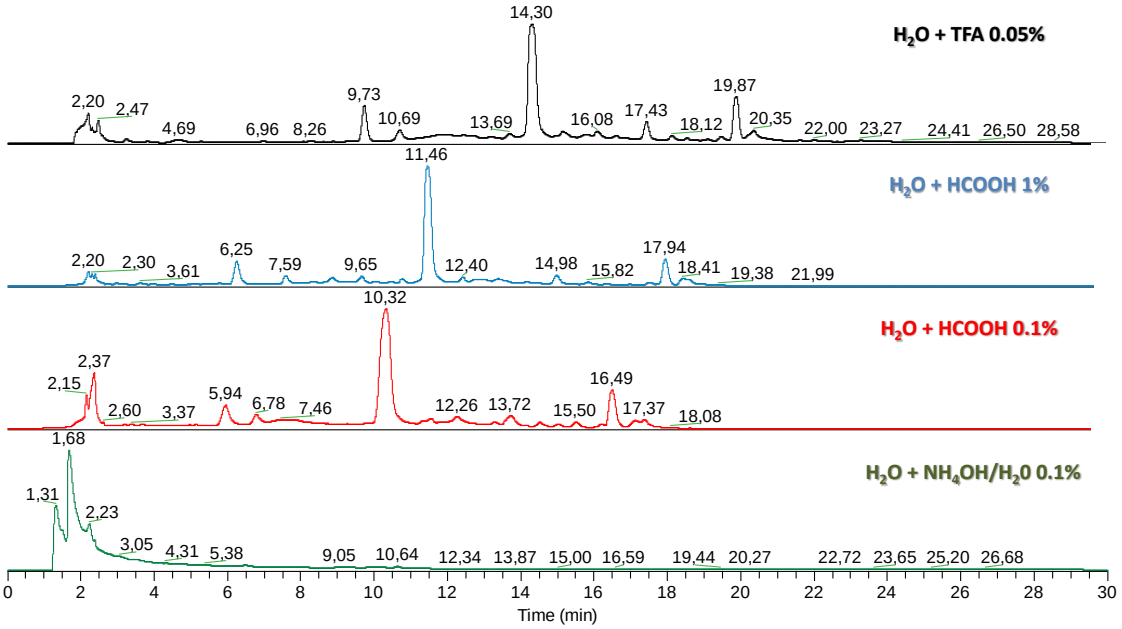


Figure 3.5 – HPLC chromatograms obtained for *Plectranthus* apimentado using method A, method B, method C and method D, respectively.

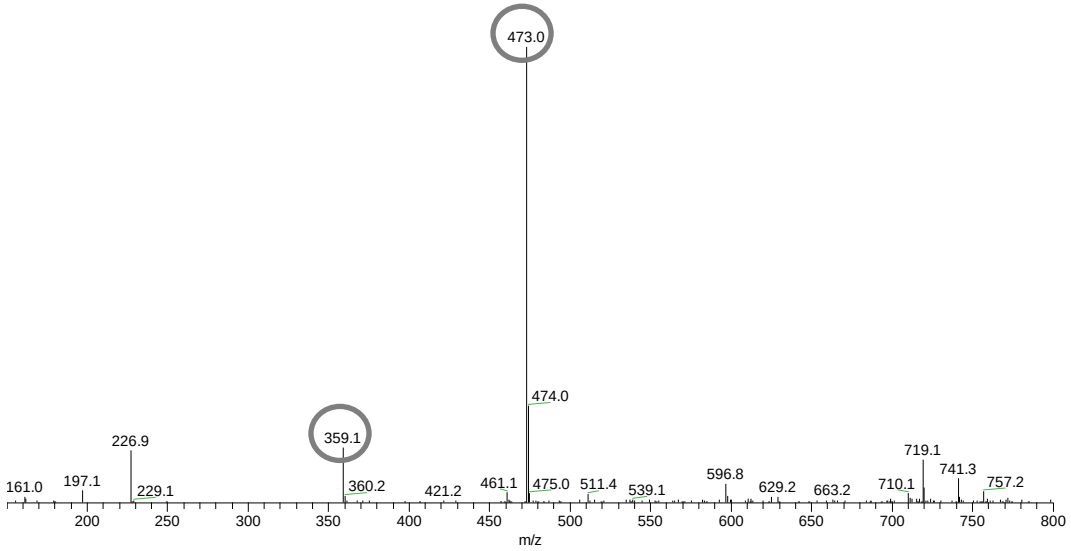


Figure 3.6 – MS spectrum of a compound present in *Plectranthus* apimentado. The compound (*m/z* 359) and the adduct formed with TFA (*m/z* 473).

Other common LC-MS eluents, such as H_2O + NH_4OH solution, were tested to see if the chromatograms' resolution improved. However, no improvement was observed (**Figure 3.5**).

The next approach consisted in adding a SPE step prior to the LC-MS^n analysis. The SPE was performed as described in chapter 2. The resulting three fractions were analysed by LC-MS^n using method C (mobile phase: MeOH and H_2O + HCOOH 0.1%). Comparing the chromatograms obtained by this approach (**Figure 3.7** to **Figure 3.9**) to the previous ones, we can verify that there were no significant differences, whereby this approach was also abandoned.

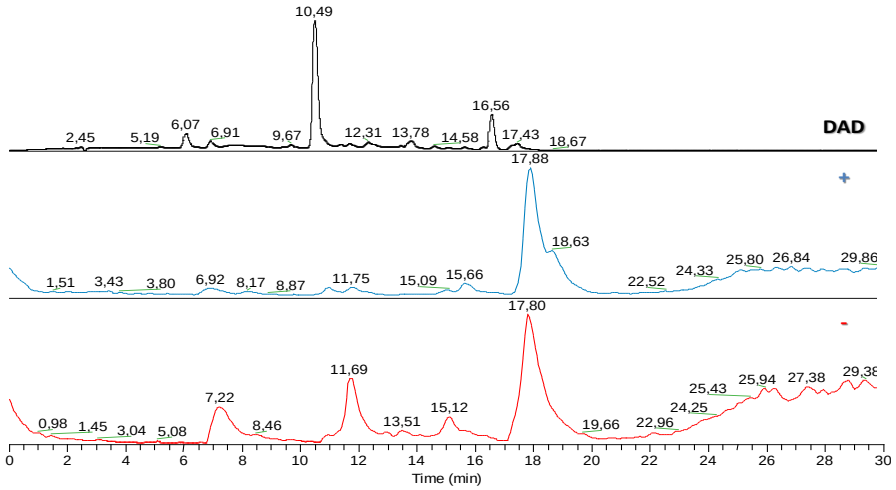


Figure 3.7 – HPLC chromatogram, LC-MS chromatogram (positive ion mode) and LC-MS chromatogram (negative ion mode) of *Plectranthus* apimentado after SPE (MeOH fraction).

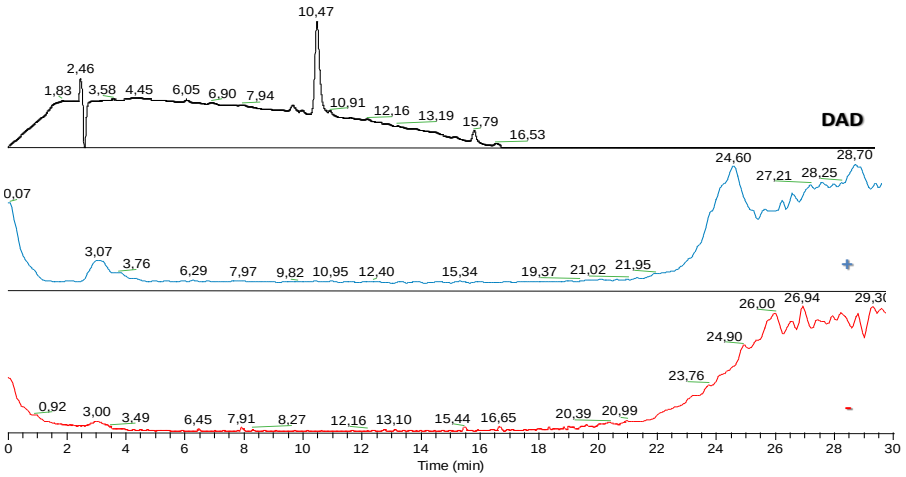


Figure 3.8 – HPLC chromatogram, LC-MS chromatogram (positive ion mode) and LC-MS chromatogram (negative ion mode) of *Plectranthus* apimentado after SPE (EtOAc fraction).

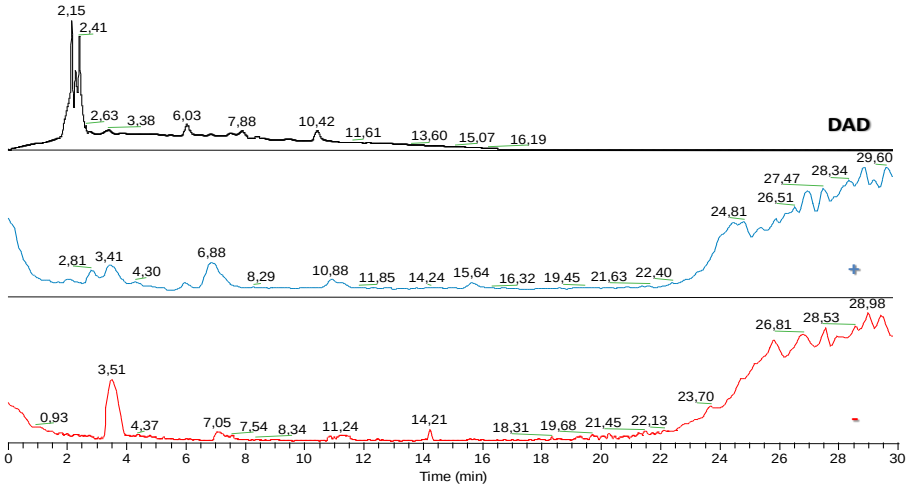


Figure 3.9 – HPLC chromatogram, LC-MS chromatogram (positive ion mode) and LC-MS chromatogram (negative ion mode) of *Plectranthus* apimentado after SPE (H_2O fraction).

Given that these are non-target LC-MS analysis, in other words, we are not targeting a single compound, but identifying all compounds possible, some changes were made to method C. We decided to increase the time of the runs from 30 minutes to 90 minutes and lower the flow rate to 0.3 mL min^{-1}, to see if more information could be gained. As can be seen in **Figure 3.10**, this approach was successful, since more compounds can now be detected.

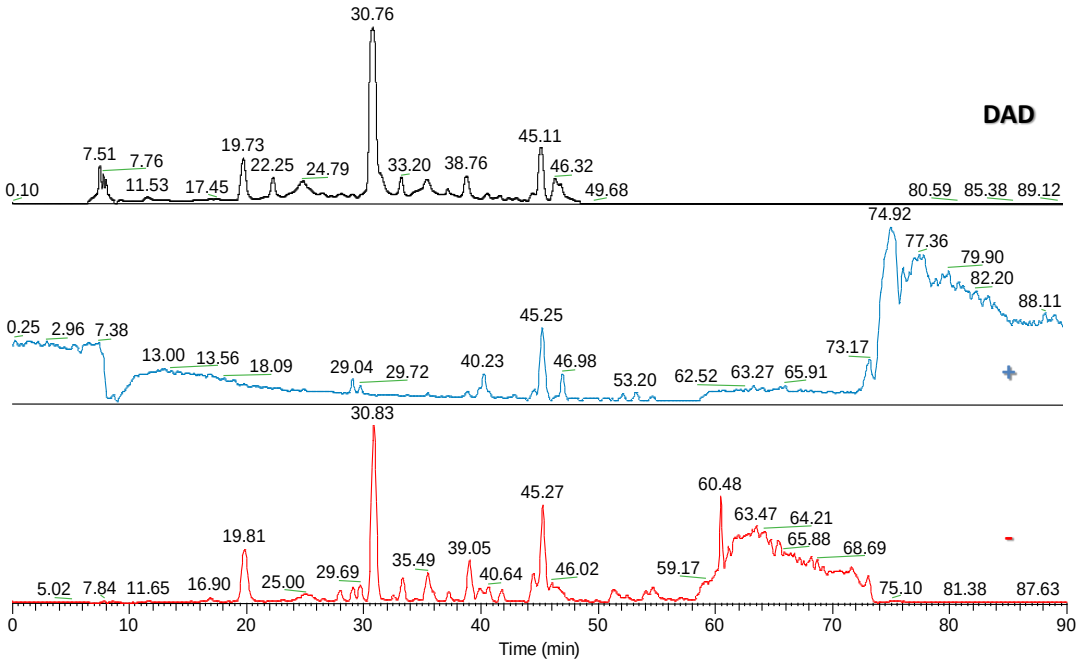


Figure 3.10 – HPLC chromatogram, LC-MS chromatogram (positive ion mode) and LC-MS chromatogram (negative ion mode) of *Plectranthus* apimentado using method E.

3.3. Identification of compounds present in aqueous extracts of *Plectranthus*

3.3.1. Identification of the compounds by LC-MS^n analysis

The identification of the compounds present in the *Plectranthus* extracts was achieved by LC-MS^n experiments and subsequent analysis of the obtained product ions. The chromatograms of each extract are presented in Appendix B and the respective MS^n values of each peak are presented in **Table 3.7** to **Table 3.16**. All ions were identified in the negative ion mode, except the ion with *m/z* 227, which was identified in the positive ion mode.

As can be seen in **Table 3.6**, there is one ion that was detected in the ten extracts. This compound, detected at around minute 31, was identified as rosmarinic acid by comparison with the MassBank database (score 0.6843). Analysing its MS spectrum, we can observe rosmarinic acid (*m/z* 359), its proton bound dimer (*m/z* 719) and its proton bound trimer (*m/z* 1079). In the MS^2 and MS^3 spectra we can see the major fragments correspond to ions at *m/z* 161, *m/z* 179 (caffeic acid) and *m/z* 197 (2-hydroxy derivative of hydrocaffeic acid) (Appendix C2). Comparing these losses to the ones described in the literature, we can confirm that this compound is rosmarinic acid.[7]

There were three ions (*m/z* 593, *m/z* 525 and *m/z* 405) that were only identified in the PA extract. The compound with *m/z* 593 was detected at 19.81 minutes and identified, by comparison of its product ions, as an apigenin diglucoside.[8] In its MS^2 spectrum we can observe losses of 90 and 120 Da (*m/z* 503 and *m/z* 473), which are characteristic for the cleavage of flavone-*C*-glucosides. In the MS^3 spectrum these losses are again observed, at *m/z* 383 and *m/z* 353 (Appendix C1). Thus, we can identify this compound as being an apigenin-*C*-diglucoside, more precisely an apigenin-6,8-*C*-diglucoside. The MS spectrum of the second ion, *m/z* 525, shows an adduct formed between the compound and HCOOH, yielding an $[M+HCOO^-]^-$ ion. In the MS^2 spectrum this adduct is broken, leading to the fragmentation of the ion with *m/z* 525. The two major product ions here observed correspond to losses of 162 Da and 180 Da. This suggests that the compound could have a glucose or galactose moiety. The distinction between these two isomers could be done by more detailed MS^n experiments. The ion *m/z* 345, correspondent to the aglycone, could be syringetin (a flavonol). The MS^3 spectrum shows of H_2O and CH_3 (Appendix C3), with are both possible from a syringetin molecule. Since characteristic losses of flavonoid-*C*-

glycosides (90 and 120 Da) were not found, this compound is assumed to be a syringetin-*O*-glycoside. The MS spectrum of the third ion, *m/z* 405, also shows an adduct formed between the compound and HCOOH, yielding an $[M+HCOO^-]^-$ ion, but in this case it is less abundant. In the MS^n spectra, we can observe losses of H_2O, CH_3OH and CH_3COOH (Appendix C4). However, this compound was not identified.

In the PE extract, an ion with *m/z* 717 was detected at 26.68 minutes. Losses of 44 Da (*m/z* 673), of 180 Da (*m/z* 537) and of 198 Da (*m/z* 519) were observed in the MS^2 spectrum. By fragmentation of the major product ion (*m/z* 519), two significant losses were again observed: loss of 180 Da (*m/z* 339) and loss of 198 Da (*m/z* 321) (Appendix C5). Analysing these losses, the compound was identified as lithospermic acid B.[9] Thus, the losses of 180 Da may correspond to the successive loss of caffeic acid. In continuation, the ion with *m/z* 537 (Appendix C9), detected in PL and PVEN extracts, was identified as lithospermic acid.[7, 9] The difference between this compound and lithospermic acid B is the loss of 180 Da (caffeic acid).

In the PE and PL extracts, an ion with *m/z* 445 was detected between minutes 35 and 36. In its MS spectrum, beyond the compound itself, we can also observe its proton bound dimer with *m/z* 891 (Appendix C6). In the MS^2 and MS^3 spectra, a loss of 176 Da can be observed, yielding an ion with *m/z* 269. The loss of 176 Da corresponds to the loss of glucuronic acid and is characteristic of flavonoid-*O*-glucuronides. The aglycone (*m/z* 269) is a flavone, apigenin. Thus, this compound was identified as an apigenin-*O*-glucuronide. It has already been identified in *Plectranthus barbatus* herbal tea, in the positive ion mode, as apigenin-7-*O*-glucuronide.[10]

In the PL extract, an ion was detected at 35.72 minutes with *m/z* 475. As in the previous case, a loss of 176 Da was observed, which indicates that we dealing with a flavonoid-*O*-glucuronide. In the MS^2 and MS^3 spectra, we can see the aglycone (*m/z* 299) and a subsequent loss of 15 Da (*m/z* 284) (Appendix C11). The *m/z* 299 corresponds to a flavone, chrysoeriol, which has already been analysed during this work. Thus, this compound was identified as chrysoeriol-*O*-glucuronide.

An ion with *m/z* 673 was detected in the PE and PVEN extracts. The MS^n spectra show losses of 18 Da ($-H_2O$), of 28 Da (-CO) and of 44 Da ($-CO_2$) (Appendix C7). This compound was not identified. However, according to the observed losses, we can assume that it is a phenolic compound.

Table 3.6 – *m/z* values of the compounds found in each extract of *Plectranthus*. (+ = detected)

All ions were detected in the negative ion mode, except the ion *m/z* 227 that was identified in the positive ion mode.

m/z Extracts	593 (1)	359 (2)	525 (3)	405 (4)	717 (5)	445 (6)	673 (7)	387 (8)	537 (9)	541 (10)	475 (11)	431 (12)	710 (13)	678 (14)	524 (15)	227 (16)	421 (17)	511 (18)
PA	+	+	+	+														
PE		+			+	+	+											
PG		+																
PL		+				+			+	+	+							
PM		+								+			+	+	+	+		
PN		+											+	+				+
PV		+											+	+	+			
PVEN		+					+		+						+	+		
PVUB		+										+					+	
PZ		+						+					+	+				

The PZ extract showed an ion with *m/z* 387, detected at 19.56 minutes. In the MS spectrum, its proton bound dimer could also be observed at *m/z* 775 (Appendix C8). Although only the MS and MS^2 spectra could be found in the literature, this compound is assumed to be medioresinol, a phytochemical usually found in the Lamiaceae family.

In the PL and PM extracts, an ion was detected around minute 26 with *m/z* 541. Although this compound was not identified, some assumptions can be made. Losses of 180 and 198 Da (*m/z* 329 and *m/z* 311) were observed in this case, similar to the ones observed with lithospermic acid and lithospermic acid B. Other ions observed were *m/z* 197 and *m/z* 179 (Appendix C10), also observed in the rosmarinic acid fragmentation. Thus, it can be assumed that this compound (*m/z* 541) could be a caffeic acid ester/derivative, as are the previous ones.

The peak detected in the PVUB extract at retention time 35.93 minutes presented an ion with *m/z* 431. The MS^n spectra showed a loss of 162 Da corresponding to the loss of a sugar moiety, yielding an ion with *m/z* 269 (aglycone), which has already been identified as apigenin (Appendix C12). Thus, this compound was identified as an apigenin-*O*-glucoside. By later comparison of these observed ions with the literature, the compound in question was assumed to be an apigenin-7-*O*- glucoside.[11]

In four extracts (PM, PN, PV and PZ), an ion with *m/z* 710 was detected between minutes 36 and 37 (Appendix C13), and another one was detected between minutes 40 and 41, with *m/z* 678 (Appendix C14). Both these compounds remain unidentified.

An ion with *m/z* 524 was detected in three extracts (PM, PV and PVEN). The MS^2 spectrum showed losses of 162 and 180 Da (*m/z* 362 and *m/z* 344) (Appendix C15), which indicate the presence of a sugar moiety. In the MS^3 spectrum, losses of H_2O, CH_3, CO and CO_2 were observed. By comparison of these product ions with the literature, this compound could be identified as a deacetylisoipecoside.[12]

An important ion, with *m/z* 227, was detected around minute 47 in the PM and PVEN extracts. It corresponds to the most abundant peak in the chromatograms of both of these extracts. This ion was only detected in the positive ion mode, which can suggest that the molecule has nitrogen atoms (N) in its composition. This was verified by analysing its MS^3 spectrum, where successive losses of 14 Da could be observed (Appendix C16). Other observed losses were of H_2O, -OH and CH_3. However, this compound was not yet identified.

In the PVUB extract, an ion with *m/z* 421 was detected at 40.47 minutes. The MS spectrum shows the ion itself and the adduct that it forms with HCOOH, yielding a

$[M+HCOO^-]^-$ ion at *m/z* 467 (Appendix C17). The MS^n spectra showed a major product ion with *m/z* 289 (loss of 132 Da, corresponding to a pentose moiety) and another product ion with *m/z* 161. By comparison with the literature, this compound was identified as a catechin pentoside[13], a plant secondary metabolite belonging to the flavanol class.

The last ion, *m/z* 511, was detected In the PN extract at 43.47 minutes. The MS^2 spectrum showed losses of 18 Da/-H_2O (*m/z* 493) and of 44 Da/-CO_2 (*m/z* 467) (Appendix C18). In the MS^3 spectrum we can see the product ions from the fragmentation of *m/z* 467. The major product ion (*m/z* 305) corresponds to the loss of 162 Da, suggesting the presence of a sugar moiety. However, this compound was not identified.

The structures of all the identified compounds are shown in **Figure 3.11**.

3.3.2. Comparison between the percentages of rosmarinic acid present the extracts and their antioxidant potential

Rosmarinic acid is known to possess a large number of interesting biological activities, including a good antioxidant activity[14]. As mentioned above, RA was the only compound detected in the ten extracts studied and was the most abundant one in them all. In **Figure 3.12**, we can see the % of RA found in each extract. As is shown, the extract with the highest % of RA was PN, immediately followed by PV. These extracts were constituted almost only by rosmarinic acid. The extracts with the least RA were PM (around 40%) and PVEN (about 30%).

The antioxidant activity of each extract was determined using the DPPH assay. The DPPH free radical method is an antioxidant assay based on electron transfer, which produces a violet solution in ethanol. When this free radical is reduced in the presence of an antioxidant molecule, the ethanol solution turns colourless. The use of this assay provides an easy and quick way to evaluate antioxidants by spectrophotometry.[15] The antioxidant potential of a substance can then be measured by its IC50, which corresponds to the quantity of the substance that is necessary to inhibit a given biological process by half. So, the higher the IC50 value, less antioxidant potential has the substance, because more quantity of it is needed. The IC50 value for each of the *Plectranthus* extracts in presented in **Figure 3.13**.

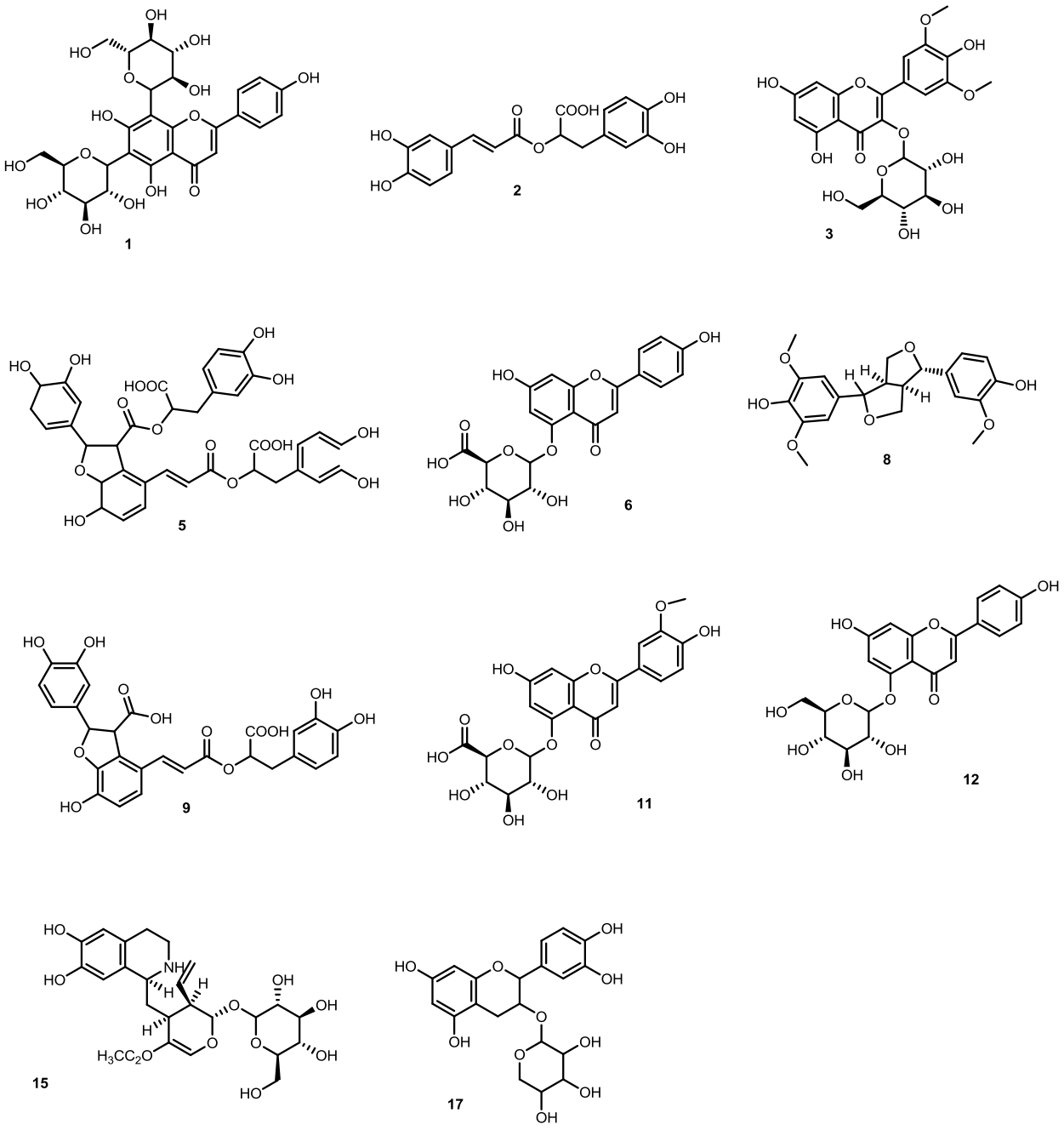


Figure 3.11 – Structures of the identified compounds present in the *Plectranthus* species.

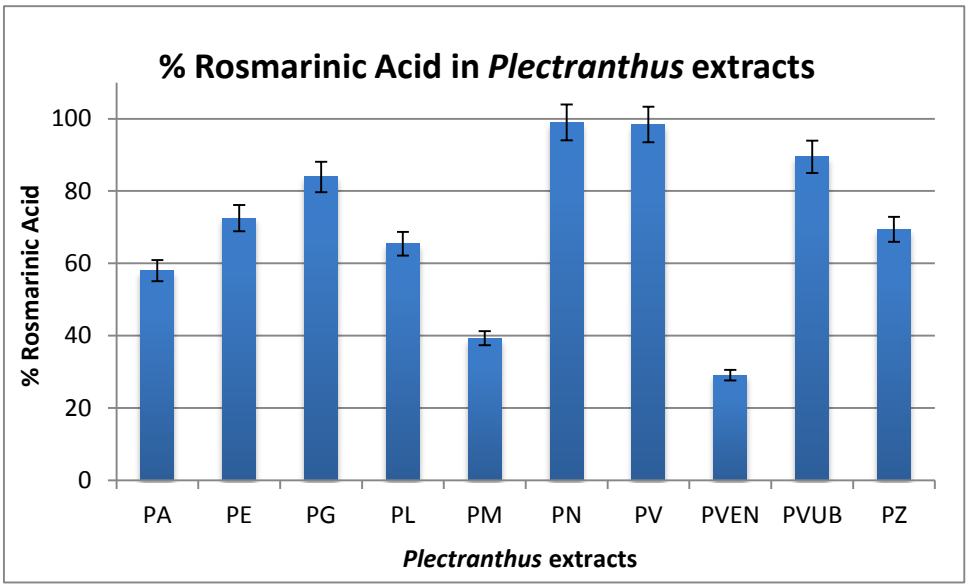


Figure 3.12 – Percentage of rosmarinic acid present in each extract of *Plectranthus*.

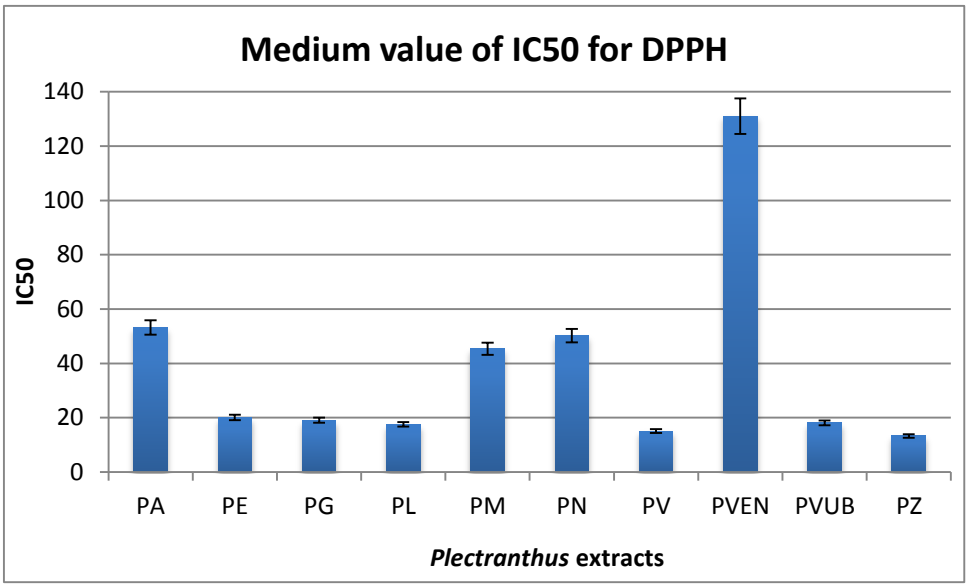


Figure 3.13 – Medium value of IC50 for DPPH of each extract of *Plectranthus*.

Taking all this into account, the extracts with higher % of RA should have a lower IC50 value, being a better antioxidant. However, as can be seen in **Figure 3.12** and **Figure 3.13**, it is not so linear. The PV, PVUB, PG, PE, PA and PM extracts are consistent with the expected, wherein the PVEN extract has less % of RA and is the extract that demonstrates less antioxidant potential. There is one extract (PN), which although it has the highest % of RA, it shows very little antioxidant activity when compared to the other extracts. On the other side, there are three extracts (PL, PM and PZ) that reveal more antioxidant activity that expected. These variations could occur due to interactions between the different compounds, such as additive, synergistic or antagonistic effects.

Table 3.7 – Retention time, *m/z* values and name of the compounds found in the PA extract.

The underlined m/z values correspond to the isolated ions for the MS^2 and MS^3 experiments.

m/z					
rt (min)	Number	MS	MS^2	MS^3	Name
19.81	1	593	473, 503, 575	353, 383,455	Apigenin-6,8-C-diglucoside
30.83	2	719, 359	359	161, 179, 223, 197, 127, 133	Rosmarinic acid (MassBank score: 0.6843)
39.05	3	571, 525	345, 363, 525, 327	312, 327, 297, 345	Syringetin-*O*-glycoside
45.27	4	405, 387, 451	387	355, 369, 327, 387	Unidentified

Table 3.8 – Retention time, *m/z* values and name of the compounds found in the PE extract.

The underlined m/z values correspond to the isolated ions for the MS^2 and MS^3 experiments.

m/z					
rt (min)	Number	MS	MS^2	MS^3	Name
26.68	5	717	519, 537, 673, 493, 555	295, 321, 339	Lithospermic acid B
31.09	2	719, 359	359	161, 179, 223, 197, 127, 133	Rosmarinic acid (MassBank score: 0.6843)
35.90	6	891, 445	445, 269	269, 175	Apigenin-*O*-glucuronide
41.81	7	673, 629	629	611, 557, 585	Unidentified

Table 3.9 – Retention time, *m/z* values and name of the compounds found in the PG extract.

The underlined m/z values correspond to the isolated ions for the MS^2 and MS^3 experiments.

m/z					
rt (min)	Number	MS	MS^2	MS^3	Name
30.79	2	719, 359	359	161, 179, 223, 197, 127, 133	Rosmarinic acid (MassBank score: 0.6843)

Table 3.10 – Retention time, *m/z* values and name of the compounds found in the PL extract.

The underlined m/z values correspond to the isolated ions for the MS^2 and MS^3 experiments.

m/z					
rt (min)	Number	MS	MS^2	MS^3	Name
25.08	9	537, 493	493, 295, 313	295, 159, 267, 109, 173	Lithospermic acid
26.28	10	541, 509	509, 311, 497, 197	311, 267, 239, 197, 239, 285	Unidentified
30.92	2	719, 359	359	161, 179, 223, 197, 127, 133	Rosmarinic acid (MassBank score: 0.6843)
35.72	6	891, 445	445, 269	269, 175	Apigenin-*O*-glucuronide
46.59	11	475, 951	299	299, 284	Chrysoeriol-*O*-glucuronide

Table 3.11 – Retention time, *m/z* values and name of the compounds found in the PM extract.

The underlined m/z values correspond to the isolated ions for the MS^2 and MS^3 experiments.

m/z					
rt (min)	Number	MS	MS^2	MS^3	Name
26.47	10	541, 509	509, 311, 497, 197	311, 267, 239, 197, 239, 285	Unidentified
31.04	2	719, 359	359	161, 179, 223, 197, 127, 133	Rosmarinic acid (MassBank score: 0.6843)
36.44	13	710	693, 583, 356, 710, 466	356, 466, 583, 2255	Unidentified
40.65	14	723, 678	678	451, 225, 660	Unidentified
45.12	15	524	362, 313	166, 151, 195, 300, 211, 344	*N*-Deacetylisoipecoside
46.96	16	227, 209	209	149, 177, 178, 209, 194, 164, 121	Unidentified

Table 3.12 – Retention time, *m/z* values and name of the compounds found in the PN extract.

The underlined m/z values correspond to the isolated ions for the MS^2 and MS^3 experiments.

m/z					
rt (min)	Number	MS	MS^2	MS^3	Name
31.09	2	719, 359	359	161, 179, 223, 197, 127, 133	Rosmarinic acid (MassBank score: 0.6843)
36.39	13	710	693, 583, 356, 710, 466	356, 466, 583, 2255	Unidentified
40.46	14	723, 678	678	451, 225, 660	Unidentified
43.47	18	511	467, 493, 450	305, 467, 449	Unidentified

Table 3.13 – Retention time, *m/z* values and name of the compounds found in the PV extract.

The underlined m/z values correspond to the isolated ions for the MS^2 and MS^3 experiments.

m/z					
rt (min)	Number	MS	MS^2	MS^3	Name
30.91	2	719, 359	359	161, 179, 223, 197, 127, 133	Rosmarinic acid (MassBank score: 0.6843)
36.42	13	710	693, 583, 356, 710, 466	356, 466, 583, 2255	Unidentified
40.55	14	723, 678	678	451, 225, 660	Unidentified
45.20	15	524	362, 313	166, 151, 195, 300, 211, 344	*N*-Deacetylisoipecoside

Table 3.14 – Retention time, *m/z* values and name of the compounds found in the PVEN extract.

The underlined m/z values correspond to the isolated ions for the MS^2 and MS^3 experiments.

m/z					
rt (min)	Number	MS	MS^2	MS^3	Name
28.85	9	537, 493	493, 295, 313	295, 159, 267, 109, 173	Lithospermic acid
31.15	2	719, 359	359	161, 179, 223, 197, 127, 133	Rosmarinic acid (MassBank score: 0.6843)
39.04	15	524	362, 313	166, 151, 195, 300, 211, 344	*N*-Deacetylisoipecoside
45.79	7	673, 629	629	611, 557, 585	Unidentified
47.15	16	227, 209	209	149, 177, 178, 209, 194, 164, 121	Unidentified

Table 3.15 – Retention time, *m/z* values and name of the compounds found in the PVUB extract.

The underlined m/z values correspond to the isolated ions for the MS^2 and MS^3 experiments.

m/z					
rt (min)	Number	MS	MS^2	MS^3	Name
30.76	2	719, 359	359	161, 179, 223, 197, 127, 133	Rosmarinic acid (MassBank score: 0.6843)
35.93	12	431	269	269	Apigenin-*O*-glucoside
40.47	17	467, 421	421	289, 161	Catechin pentoside

Table 3.16 – Retention time, *m/z* values and name of the compounds found in the PZ extract.

The underlined m/z values correspond to the isolated ions for the MS^2 and MS^3 experiments.

m/z					
rt (min)	**Number**	**MS**	**MS^2**	**MS^3**	**Name**
19.56	8	387, 775	207, 163, 369	163	Medioresinol
31.08	2	719, 359	359	161, 179, 223, 197, 127, 133	Rosmarinic acid (MassBank score: 0.6843)
36.45	13	710	693, 583, 356, 710, 466	356, 466, 583, 2255	Unidentified
40.57	14	723, 678	678	451, 225, 660	Unidentified

3.4. References

[1] P.J. Amorim Madeira, P.D. Vaz, R.J.N. Bettencourt da Silva, M.H. Florêncio; *Can Semi-empirical Calculations Help Solve Mass Spectrometry Problems? Protonation Sites and Proton Affinities of Amino Acids*. ChemPlusChem. 78 (2013) 1149-56.

[2] H.F.P. Martins, J.P. Leal, M.T. Fernandez, V.H.C. Lopes, M.N.D.S. Cordeiro; *Toward the prediction of the activity of antioxidants: experimental and theoretical study of the gas-phase acidities of flavonoids*. Journal of the American Society for Mass Spectrometry. 15 (2004) 848-61.

[3] Y.L. Ma, Q.M. Li, H. Van den Heuvel, M. Claeys; *Characterization of flavone and flavonol aglycones by collision-induced dissociation tandem mass spectrometry*. Rapid Communications in Mass Spectrometry. 11 (1997) 1357-64.

[4] T.J. Mabry, K.R. Markham; *In The Flavonoids*; New York; Academic Press; 1975.

[5] B. Domon, C. Costello; *A systematic nomenclature for carbohydrate fragmentations in FAB-MS/MS spectra of glycoconjugates*. Glycoconjugate J. 5 (1988) 397-409.

[6] F. Cuyckens, M. Claeys; *Mass spectrometry in the structural analysis of flavonoids*. Journal of Mass Spectrometry. 39 (2004) 1-15.

[7] P.C. Dinis, P.L. Falé, P.J.A. Madeira, M.H. Florêncio, M.L. Serralheiro; *Acetylcholinesterase inhibitory activity after in vitro gastrointestinal digestion of infusions of Mentha species*. European Journal of Medicinal Plants. 3 (2013) 381-93.

[8] L. Barros, M. Dueñas, C.T. Alves, S. Silva, M. Henriques, C. Santos-Buelga, et al.; *Antifungal activity and detailed chemical characterization of Cistus ladanifer phenolic extracts*. Industrial Crops and Products. 41 (2013) 41-5.

[9] G. Zeng, H. Xiao, J. Liu, X. Liang; *Identification of phenolic constituents in Radix Salvia miltiorrhizae by liquid chromatography/electrospray ionization mass spectrometry*. Rapid Communications in Mass Spectrometry. 20 (2006) 499-506.

[10] S. Porfírio, P.L.V. Falé, P.J.A. Madeira, M.H. Florêncio, L. Ascensão, M.L.M. Serralheiro; *Antiacetylcholinesterase and antioxidant activities of Plectranthus barbatus tea, after in vitro gastrointestinal metabolism*. Food Chemistry. 122 (2010) 179-87.

[11] R.E. March, E.G. Lewars, C.J. Stadey, X.-S. Miao, X. Zhao, C.D. Metcalfe; *A comparison of flavonoid glycosides by electrospray tandem mass spectrometry*. International Journal of Mass Spectrometry. 248 (2006) 61-85.

[12] N. Taiji, Q. Alfonso Lara, M.K. Toni; *The New β-D-Glucosidase in Terpenoid-Isoquinoline Alkaloid Biosynthesis in Psychotria ipecacuanha*. Journal of Biological Chemistry. 283 (2008) 34650-9.

[13] P. Mammela; *Phenolics in selected European hardwood species by liquid chromatography-electrospray ionisation mass spectrometry*. Analyst. 126 (2001) 1535-8.

[14] M. Petersen, M.S.J. Simmonds; *Rosmarinic acid*. Phytochemistry. 62 (2003) 121-5.

[15] E.J. Garcia, T.L.C. Oldoni, S.M.d. Alencar, A. Reis, A.D. Loguercio, R.H.M. Grande; *Antioxidant activity by DPPH assay of potential solutions to be applied on bleached teeth*. Brazilian Dental Journal. 23 (2012) 22-7.

CHAPTER 4

CONCLUSIONS AND FUTURE WORK

A study of the gas-phase behaviour of five flavones (acacetin, chrysoeriol, luteolin, orientin and vitexin) was successfully performed. The most probable protonation and deprotonation sites for each flavone were determined by means of semi-empirical calculations using MOPAC2012 and the PM6 hamiltonean. While the protonation is predicted to occur at position 4 C=O group for the five flavones, the predicted deprotonation site varies: 7 OH group for acacetin, 4' OH group for chrysoeriol and luteolin, and 4 (sugar) OH group for both orientin and vitexin.

The compounds were analysed by electrospray mass spectrometry in the positive and negative ion modes. Several neutral losses were observed together with cross-ring cleavages of the aglycone and the sugar moiety, when applicable. The negative ion mode seemed to be more selective in the analysis of flavones; however the results suggest that both ion modes are complementary tools in this type of analysis.

The optimization of the chromatographic conditions enabled the best separation of the compounds present in the *Plectranthus* extracts using method E. The SPE step did not improve the chromatographic separation.

Eleven compounds were identified based on the analysis of their product ions and sequential comparison with the MassBank database and available references. However, some compounds remain unidentified.

Rosmarinic acid was the only compound present in all the extracts, consisting also in the most abundant one. The PM extract was the extract where more compounds were identified, whereas in the PG extract only one compound was identified.

The percentages of rosmarinic acid present in the extracts and their antioxidant activity revealed not to be as linear as expected, which can be due to interactions between the different compounds.

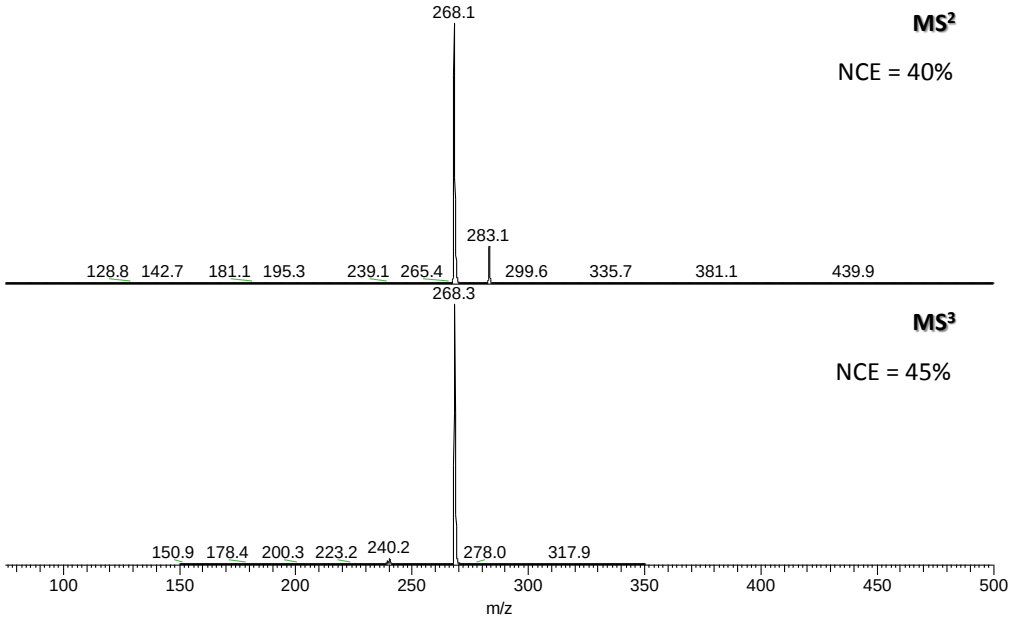
Future work will include the identification of the yet unidentified compounds, namely the ones with ions at m/z 405 and 227, that were found in relatively high percentage in the extracts of PA and PM and PVEN, respectively.

APPENDIX

A. MS^n spectra of the flavones

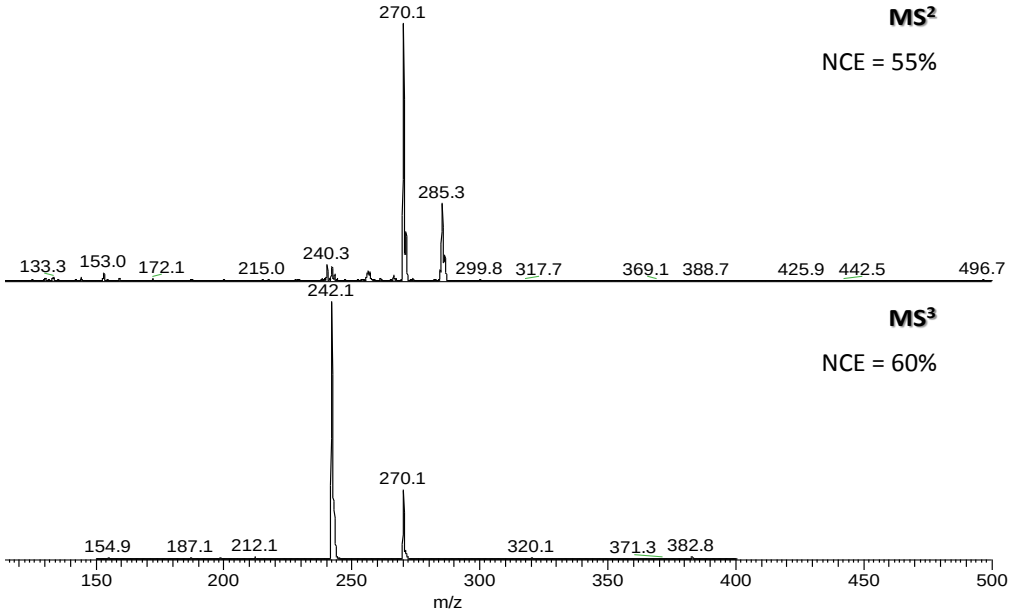
A1. Acacetin – negative ion mode

$[M-H]^-$ = 283



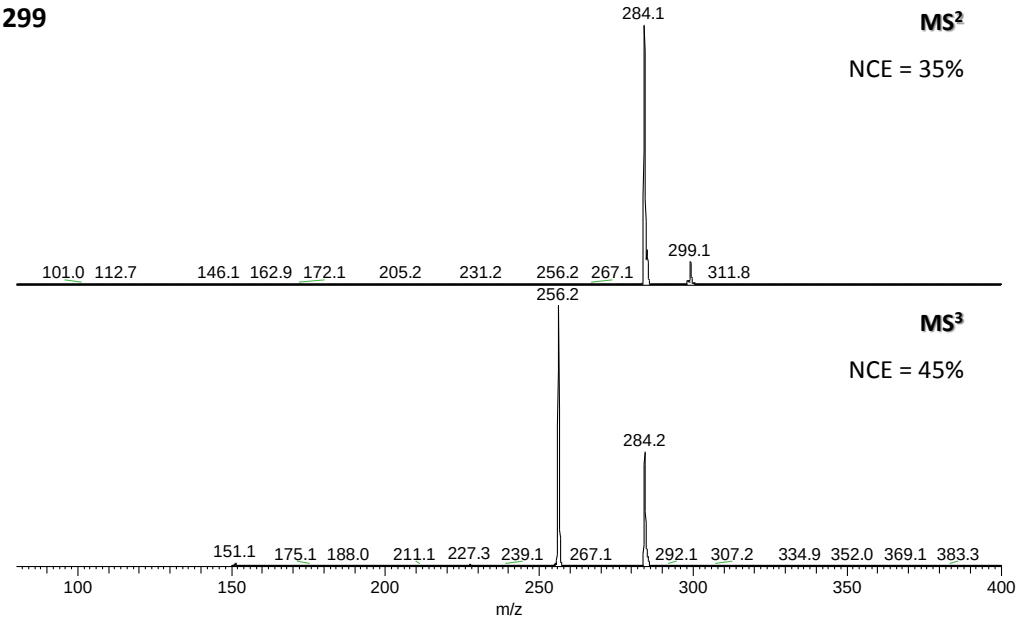
A2. Acacetin – positive ion mode

$[M+H]^+$ = 285



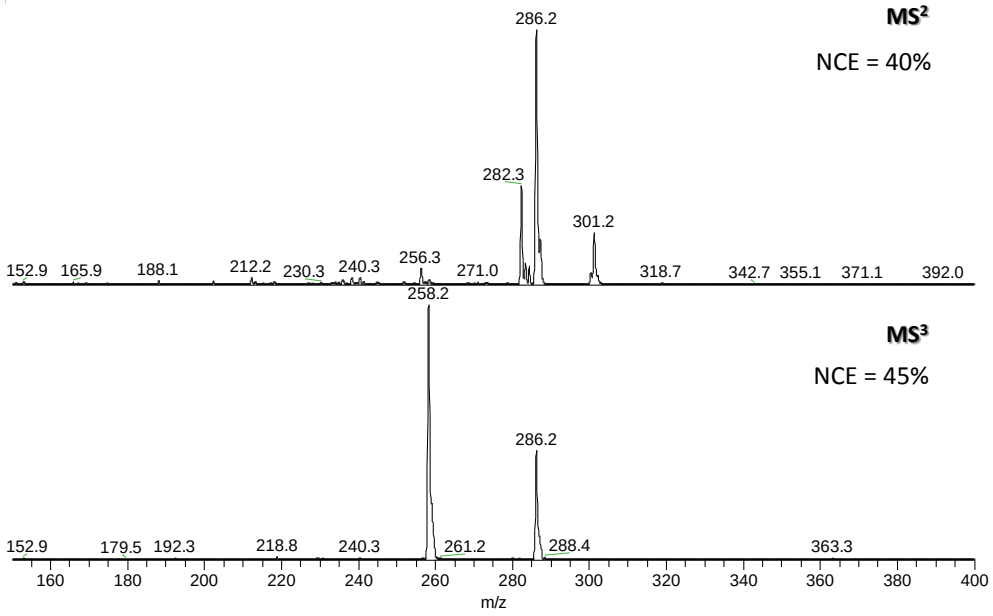
A3. Chrysoeriol – negative ion mode

$[M-H]^-$ = 299



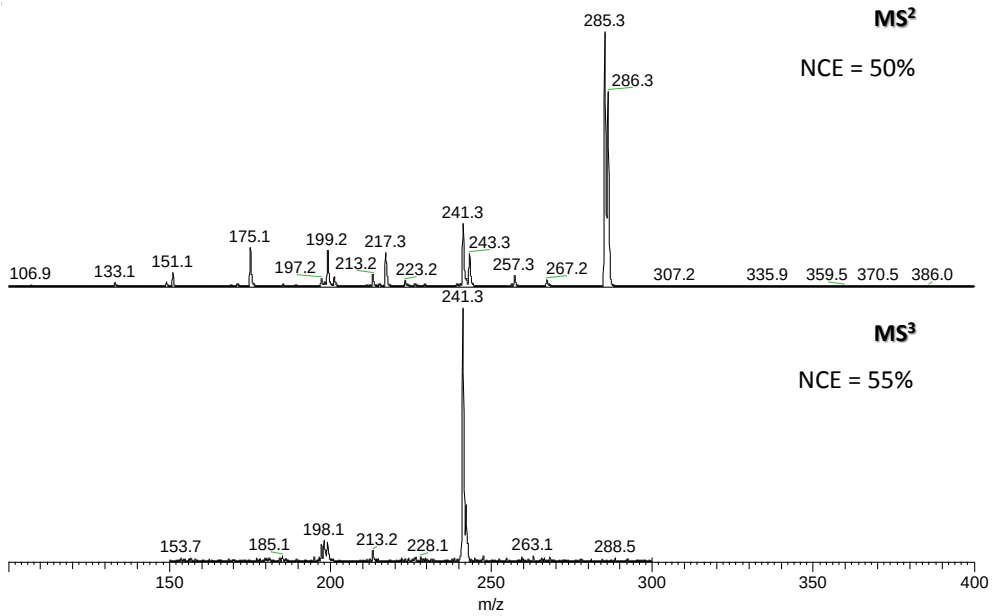
A4. Chrysoeriol – positive ion mode

$[M+H]^+$ = 301



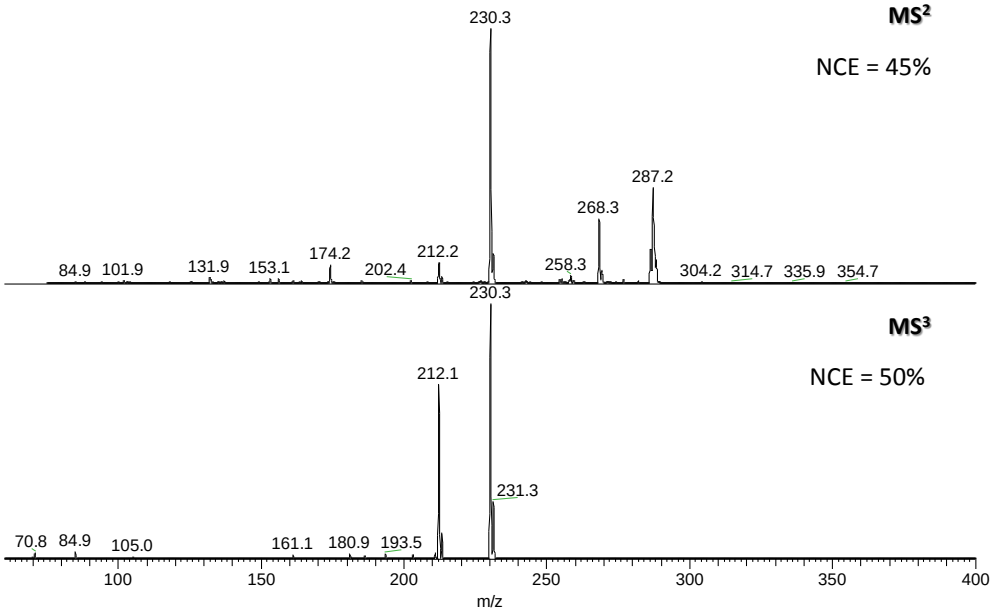
A5. Luteolin – negative ion mode

$[M-H]^-$ = 285



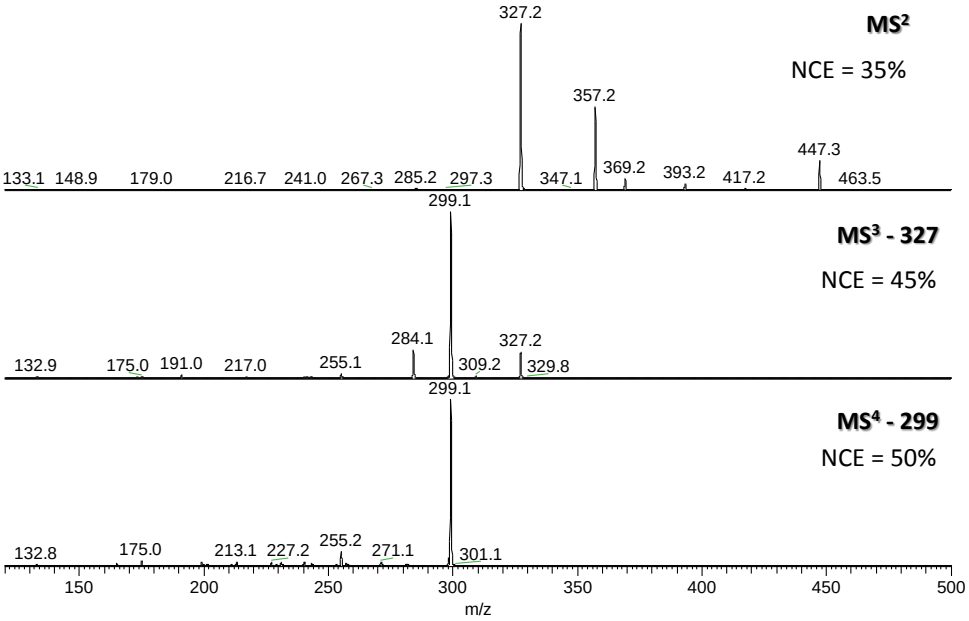
A6. Luteolin – positive ion mode

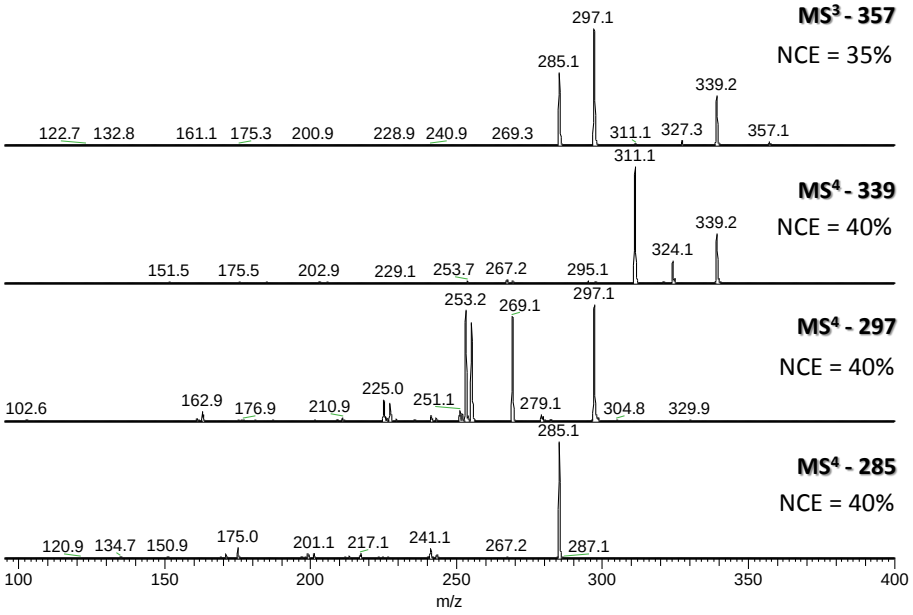
$[M+H]^+$ = 287



A7. Orientin – negative ion mode

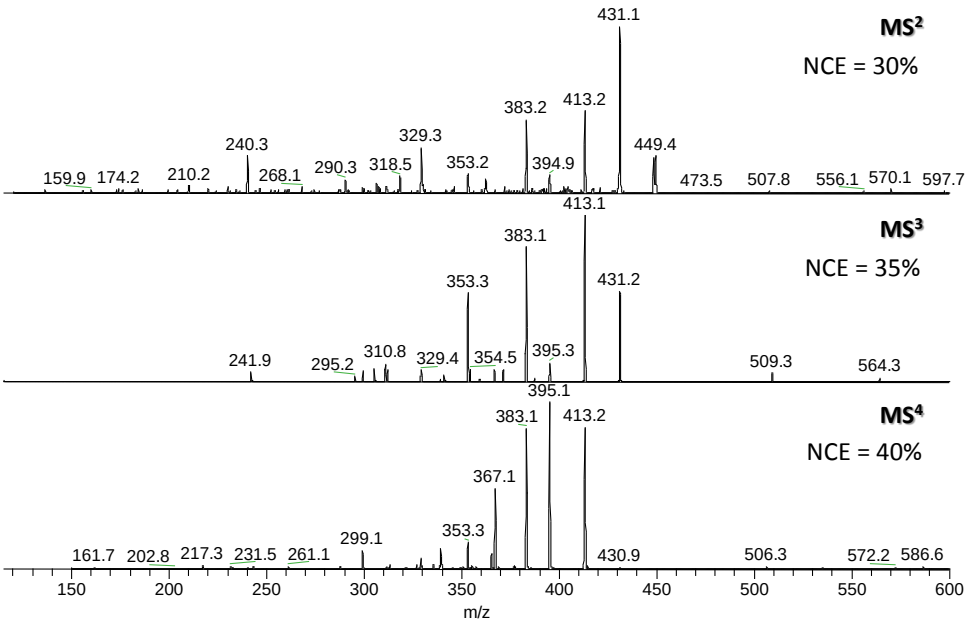
$[M-H]^-$ = 447





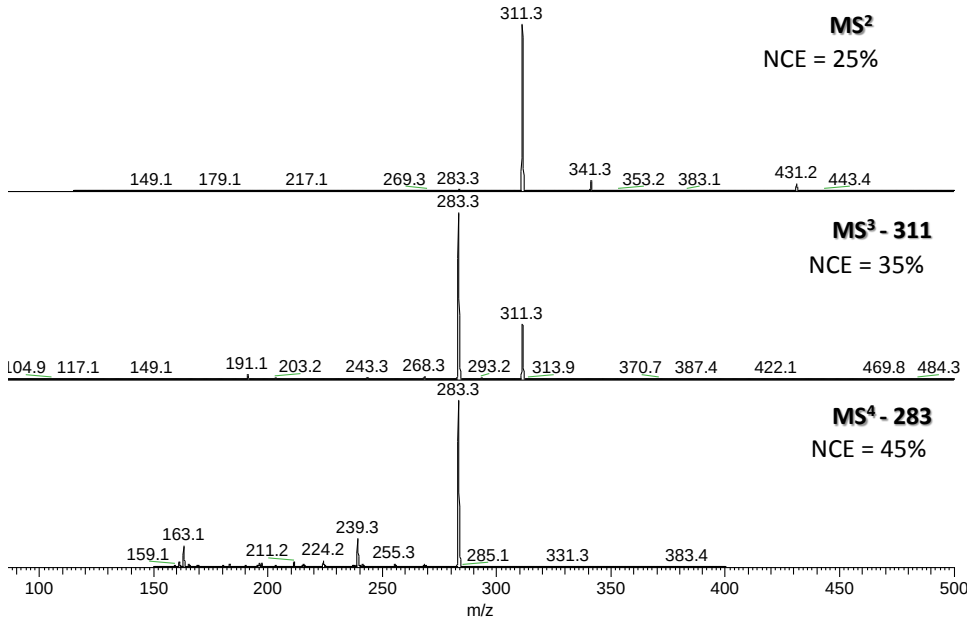
A8. Orientin – positive ion mode

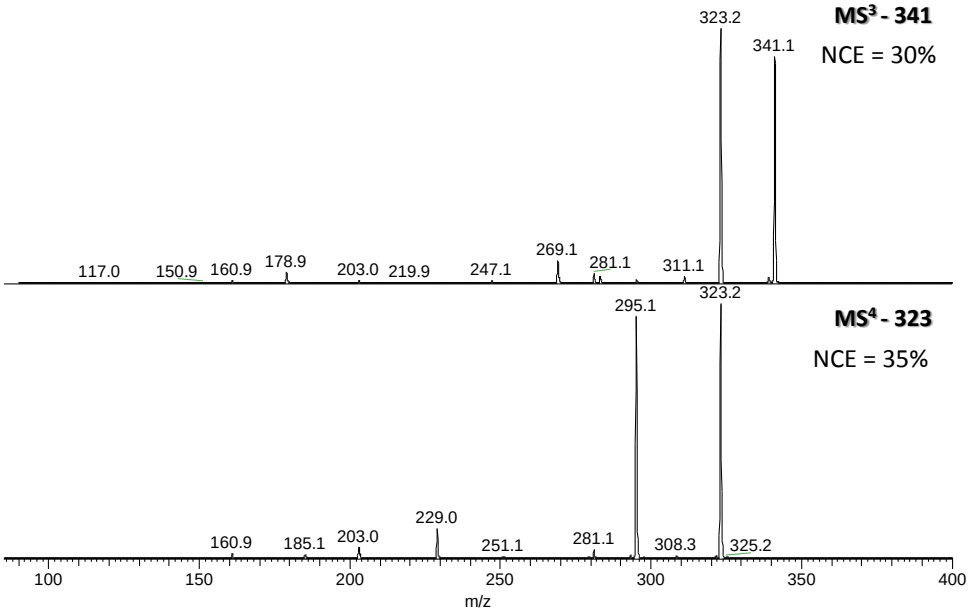
$[M+H]^+$ = 449



A9. Vitexin – negative ion mode

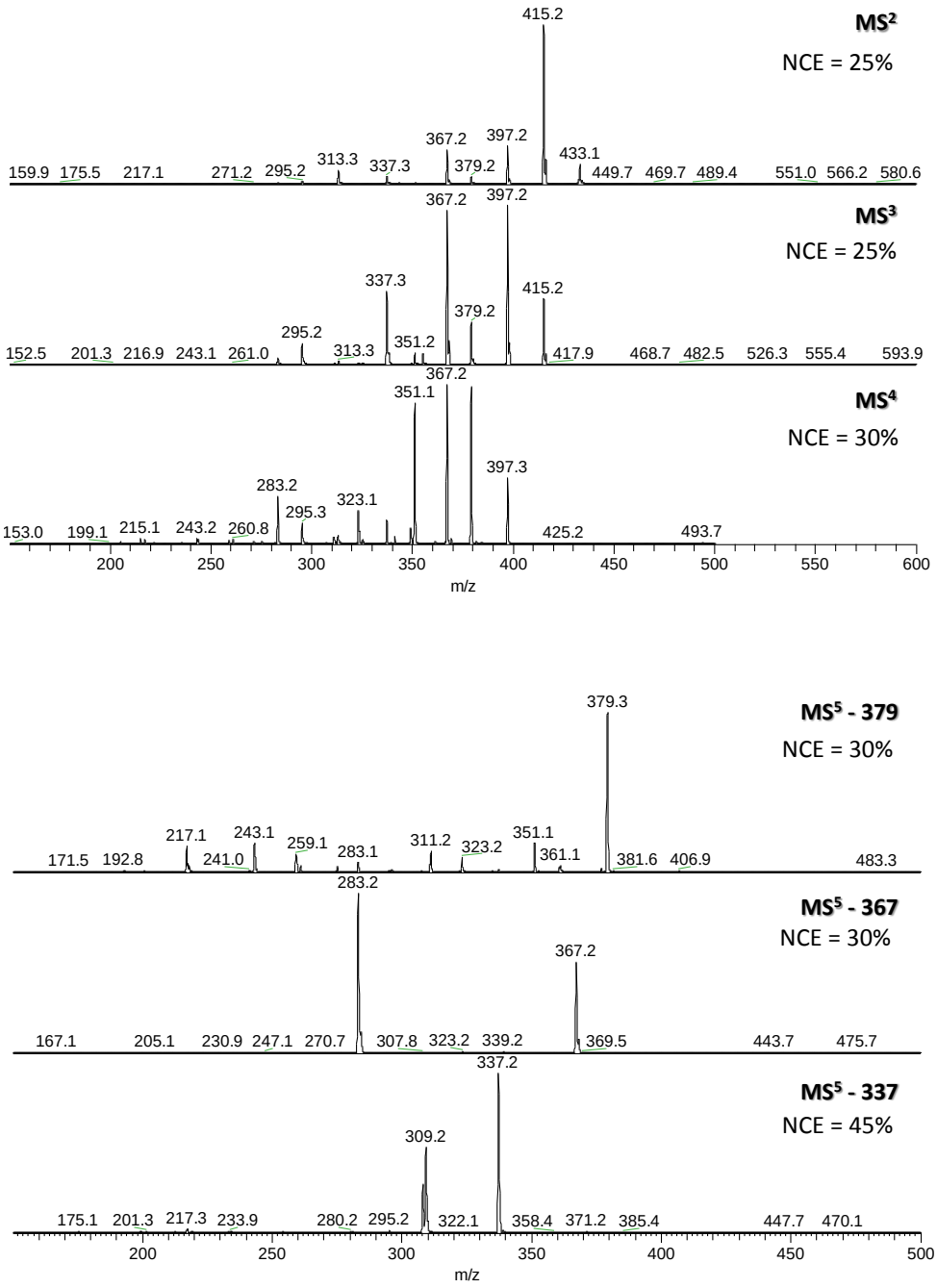
$[M-H]^-$ = 431





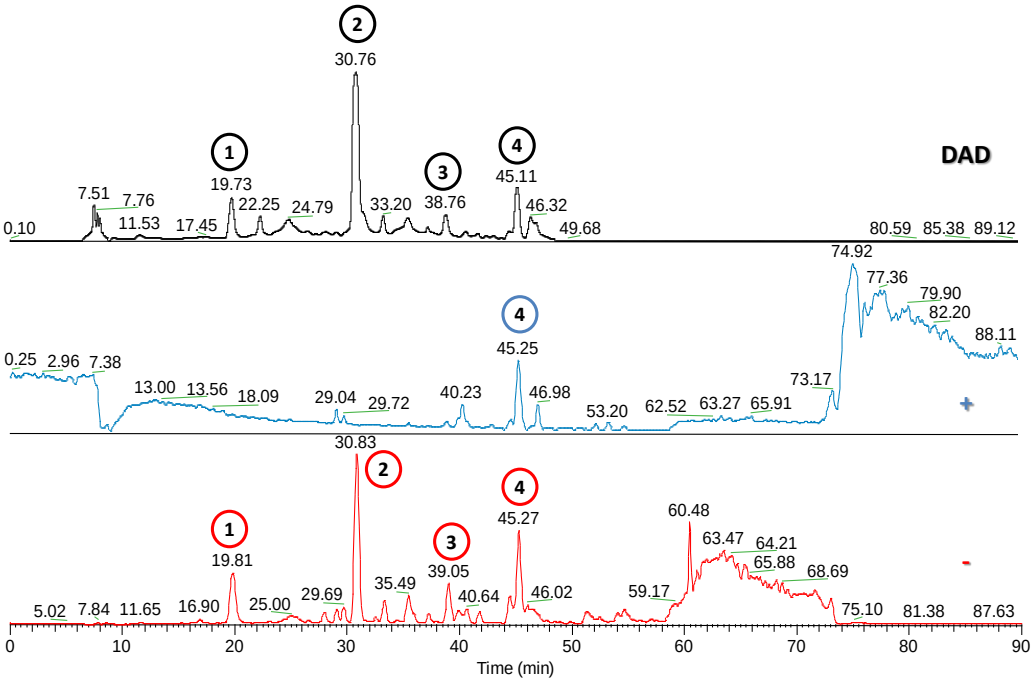
A10. Vitexin – positive ion mode

$[M+H]^+$ = 447

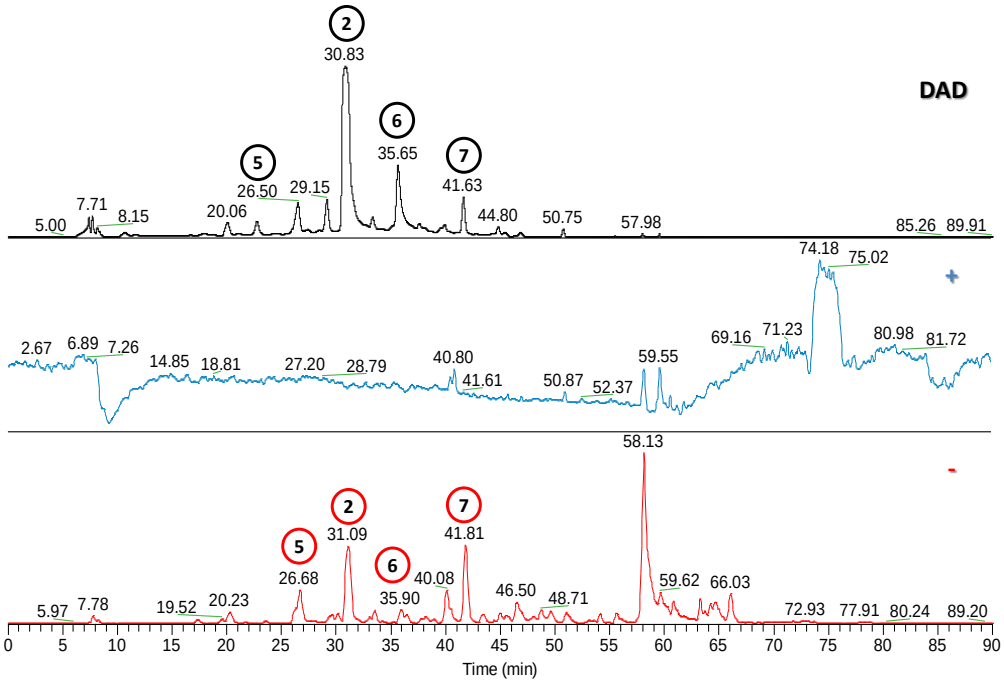


B. Chromatograms of the ten extracts

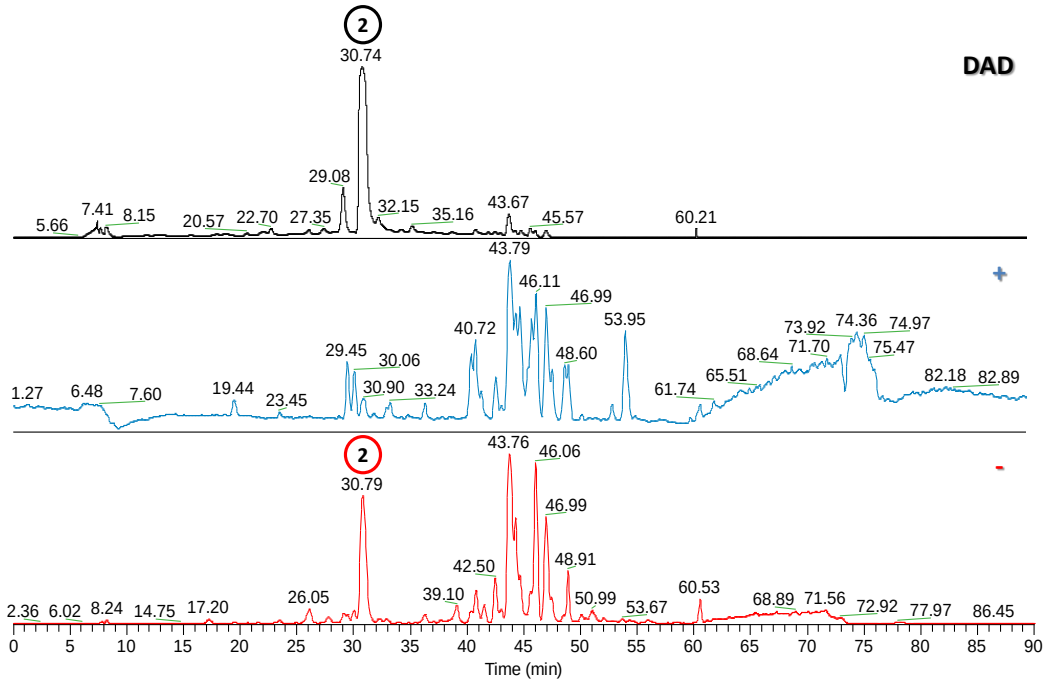
B1. PA extract



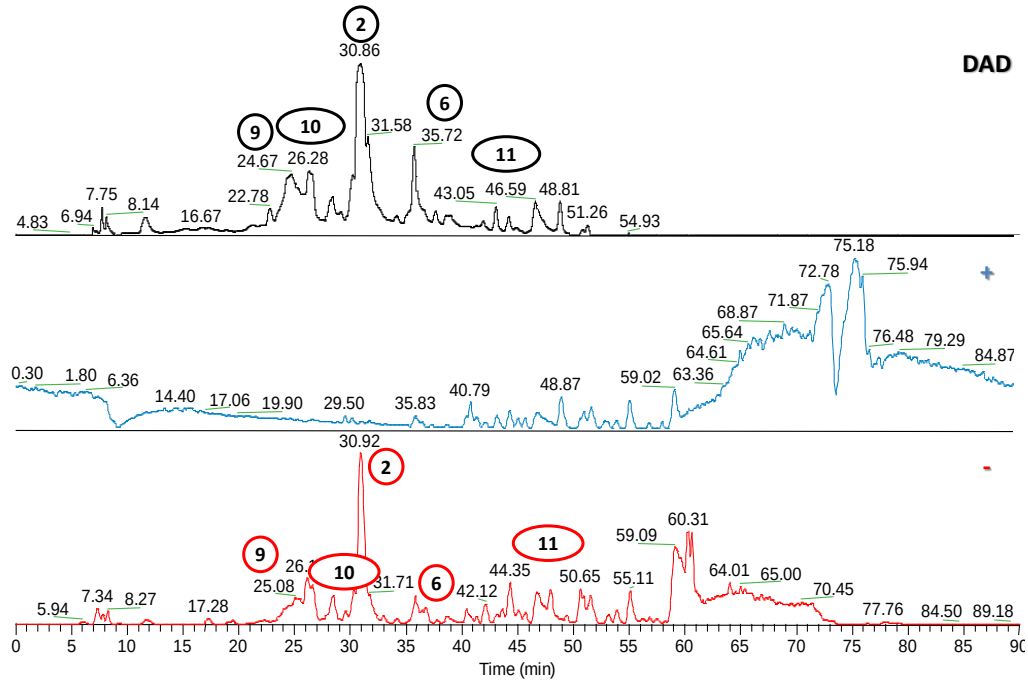
B2. PE extract



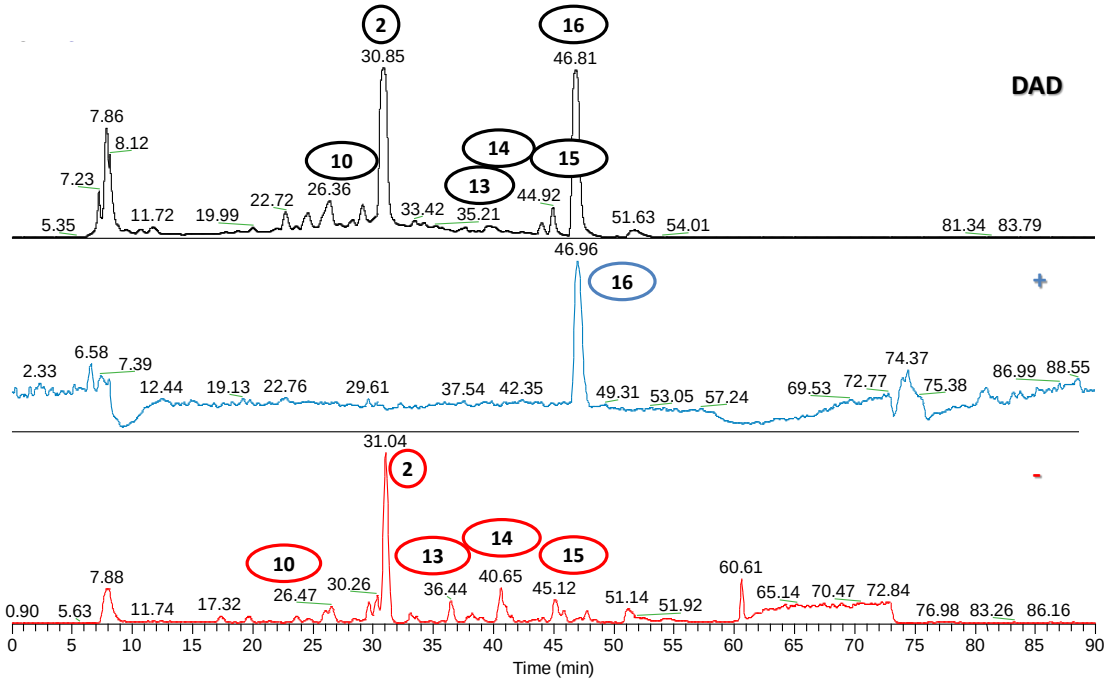
B3. PG extract



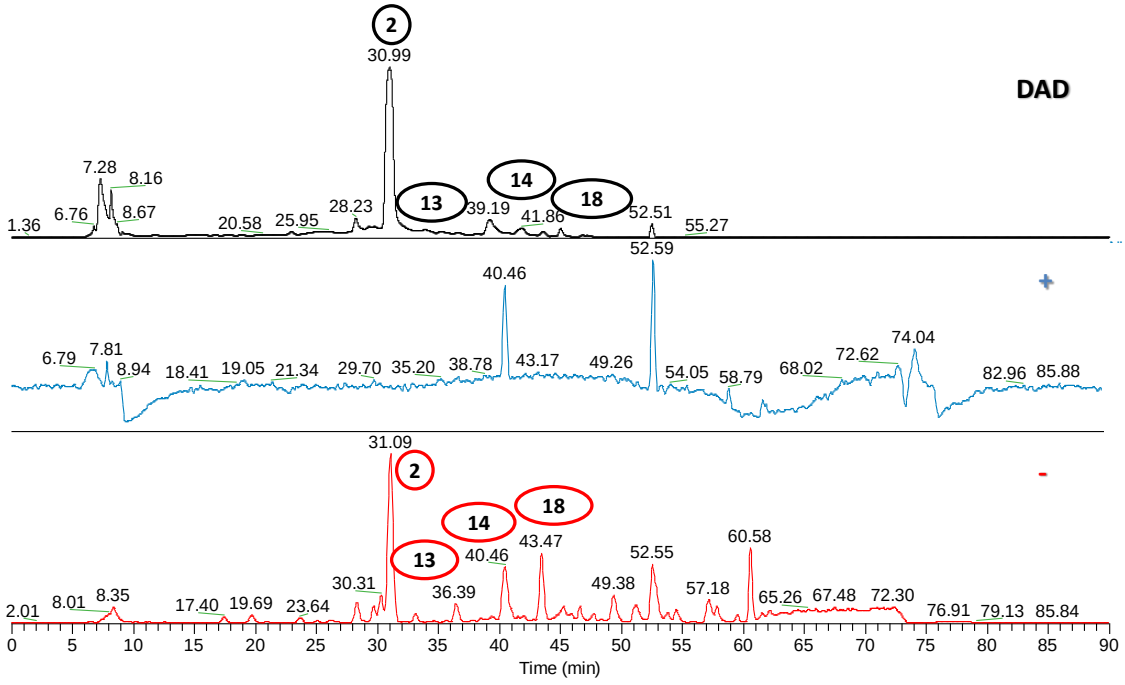
B4. PL extract



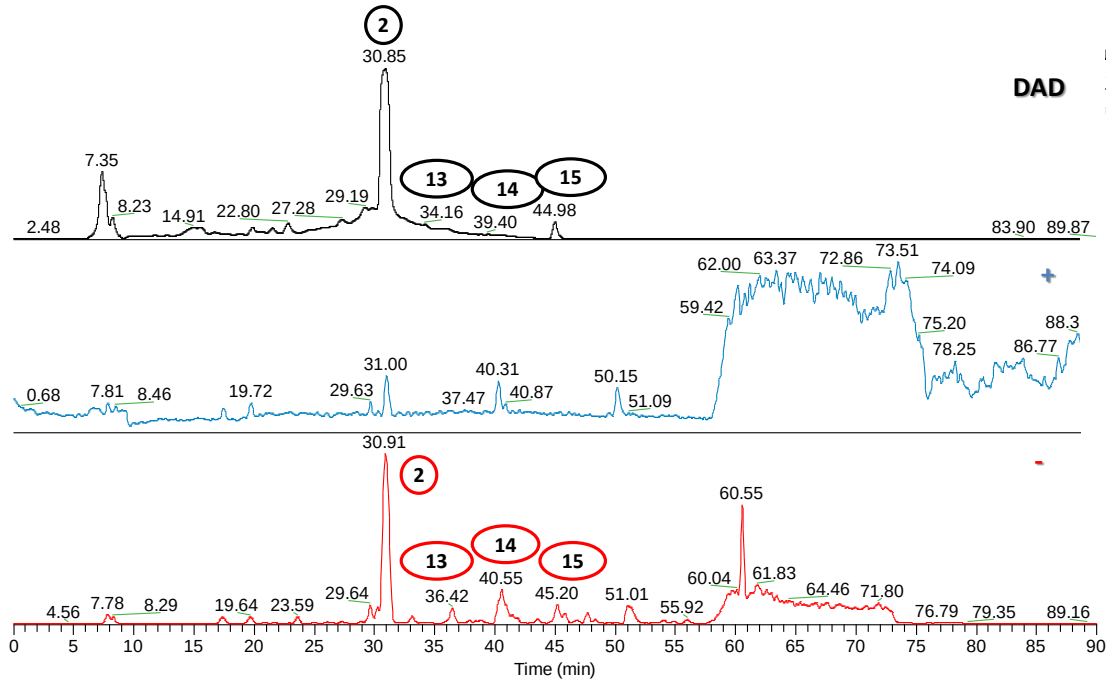
B5. PM extract



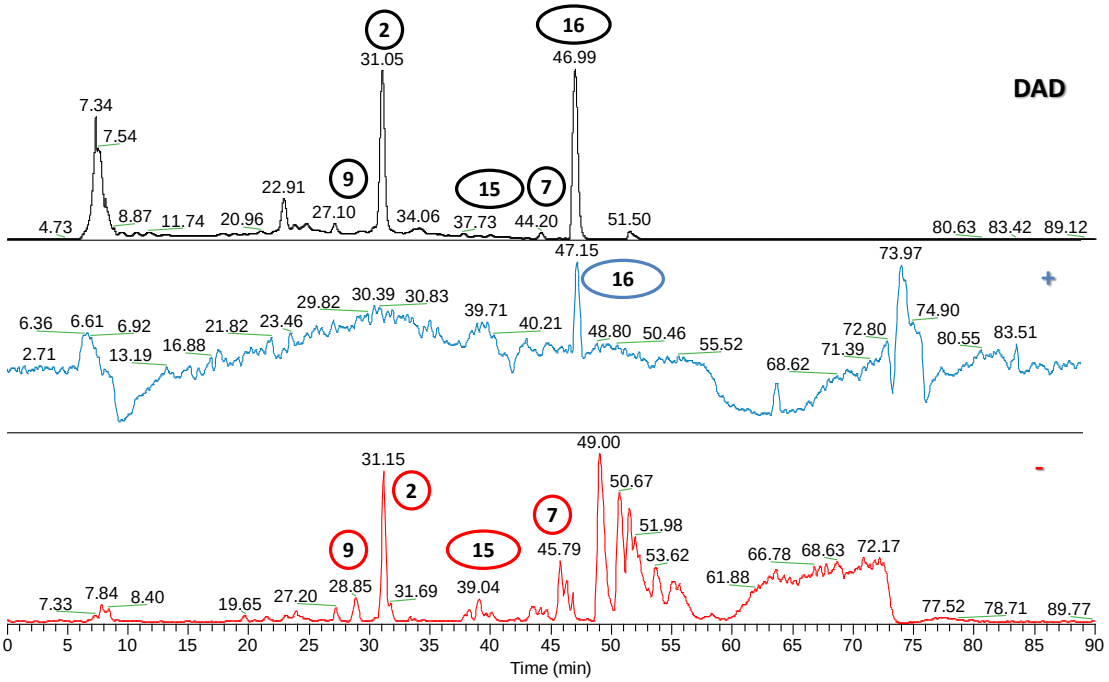
B6. PN extract



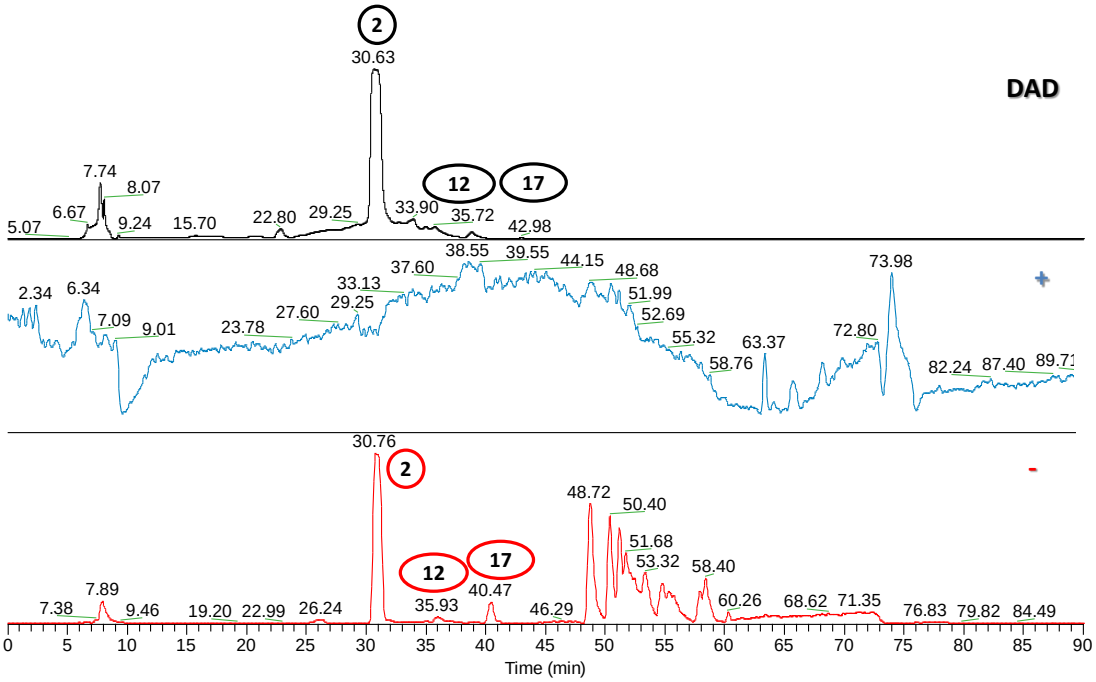
B7. PV extract



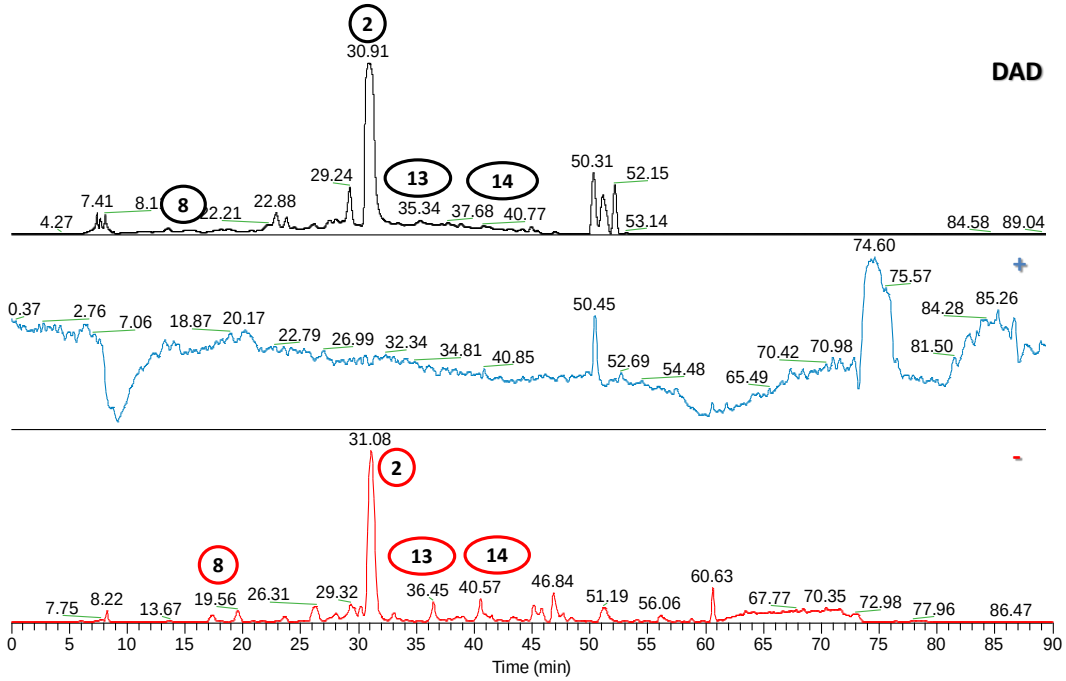
B8. PVEN extract



B9. PVUB extract

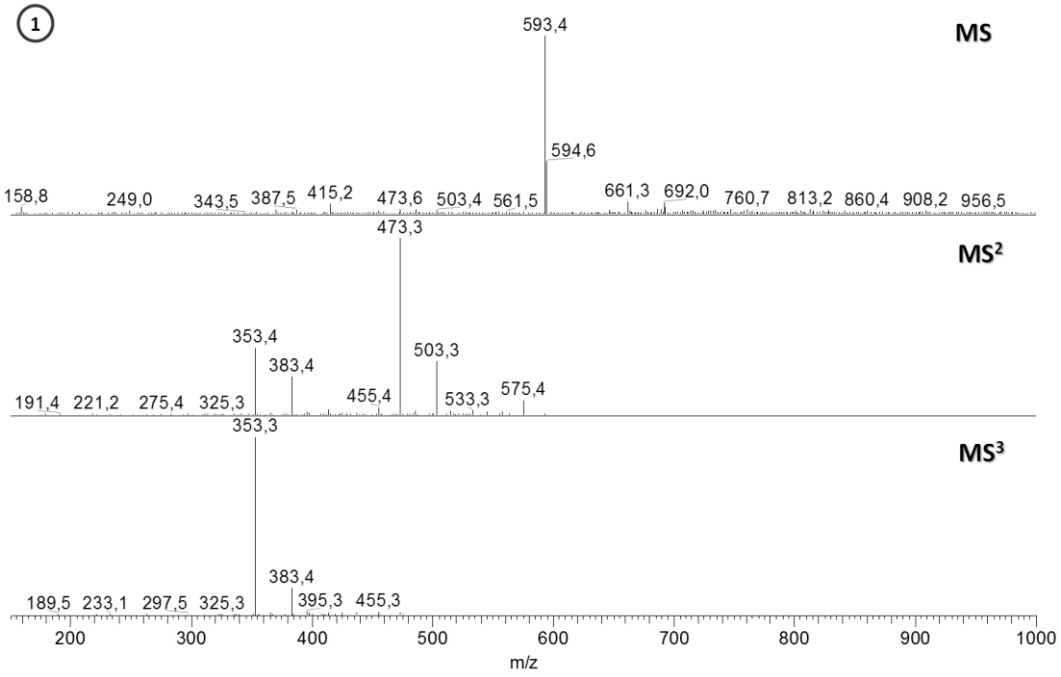


B10. PZ extract

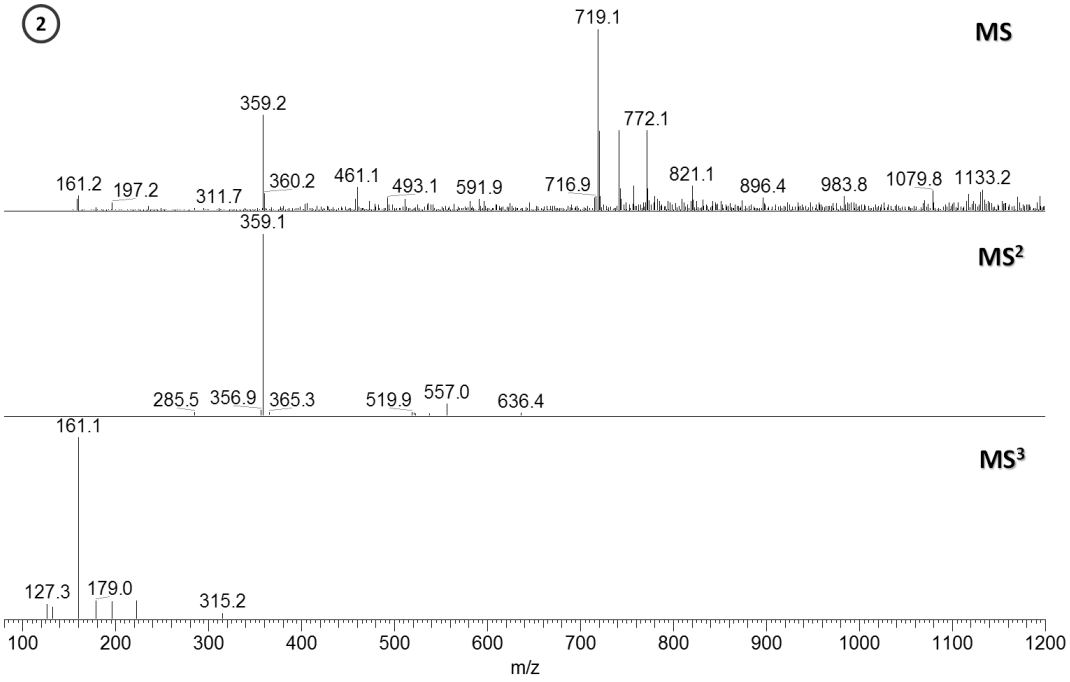


C. MS, MS^2 and MS^3 spectra of the compounds found in the aqueous extracts of *Plectranthus*

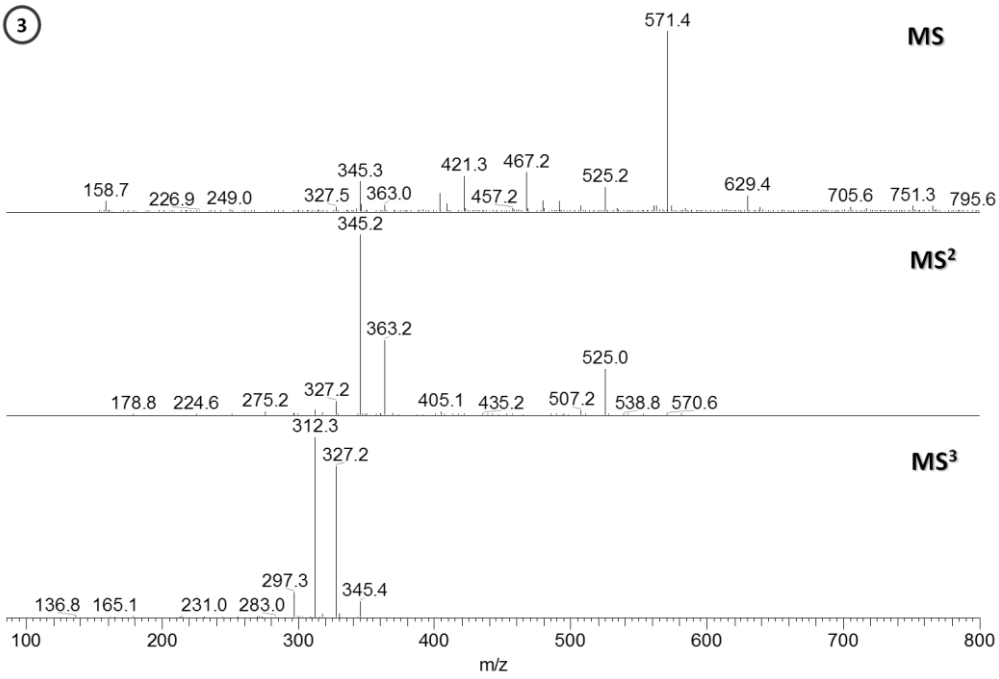
C1. Compound 1



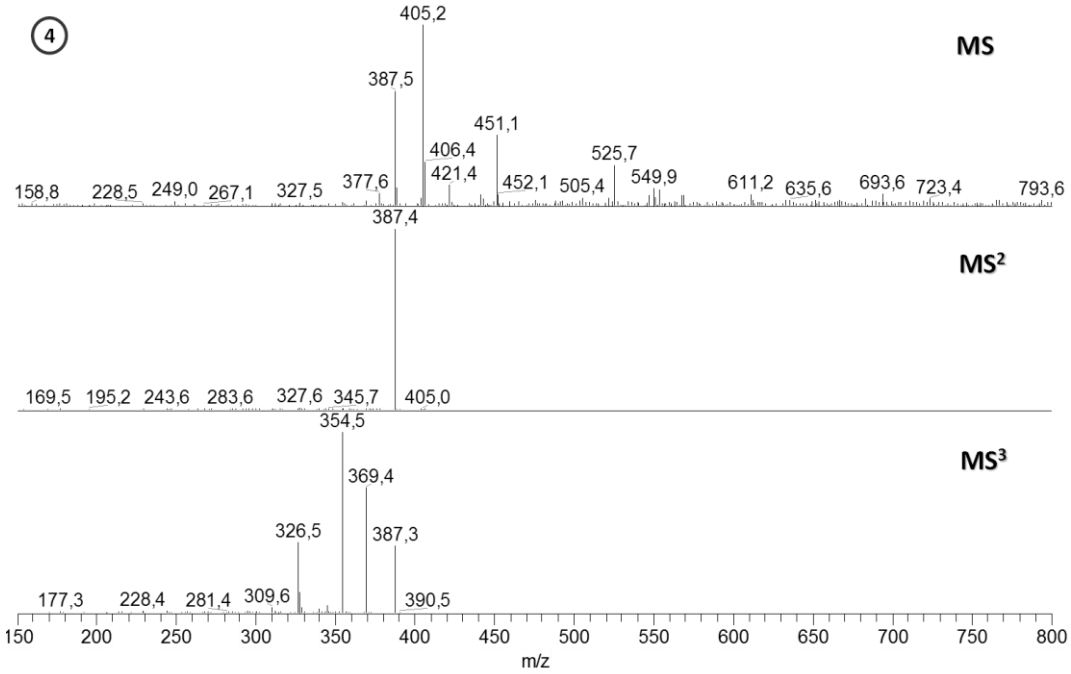
C2. Compound 2



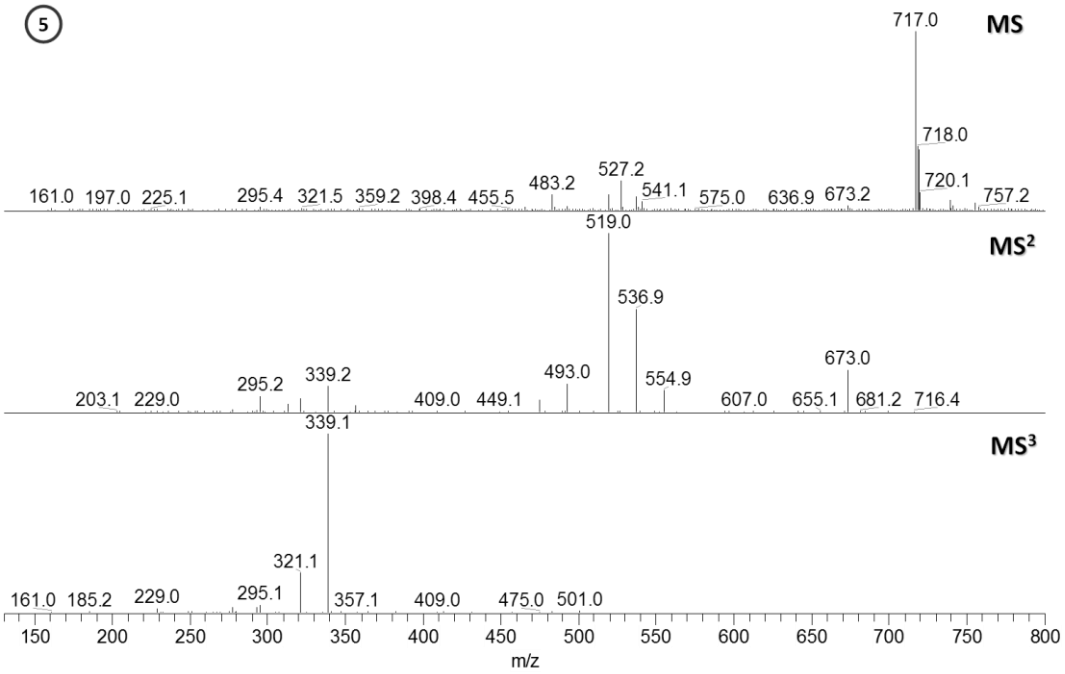
C3. Compound 3



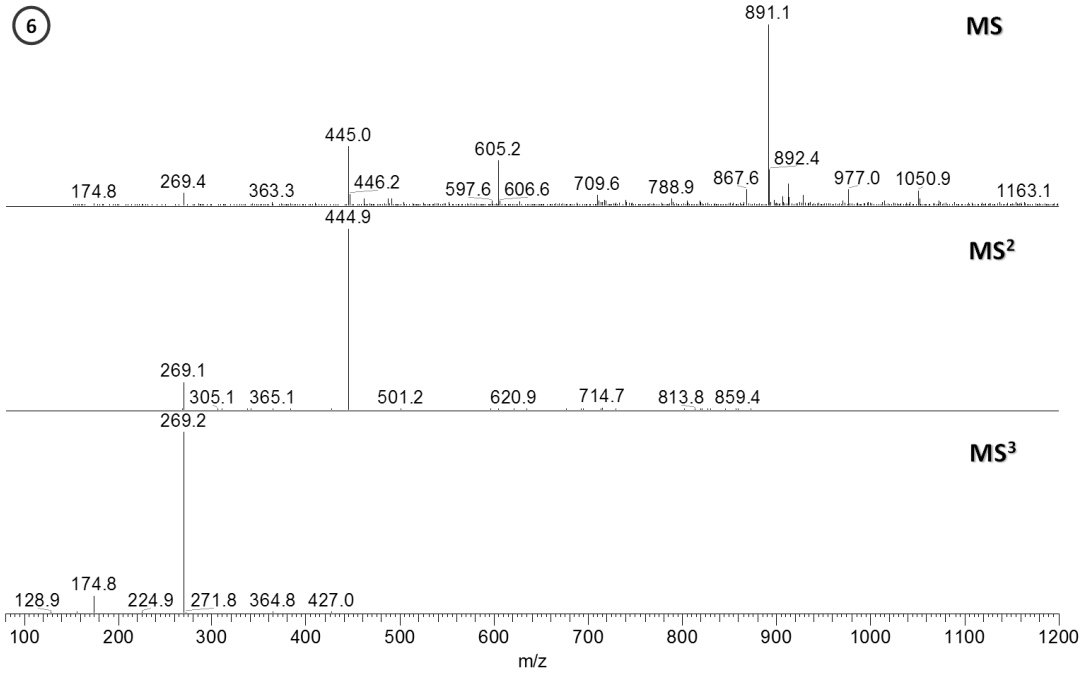
C4. Compound 4



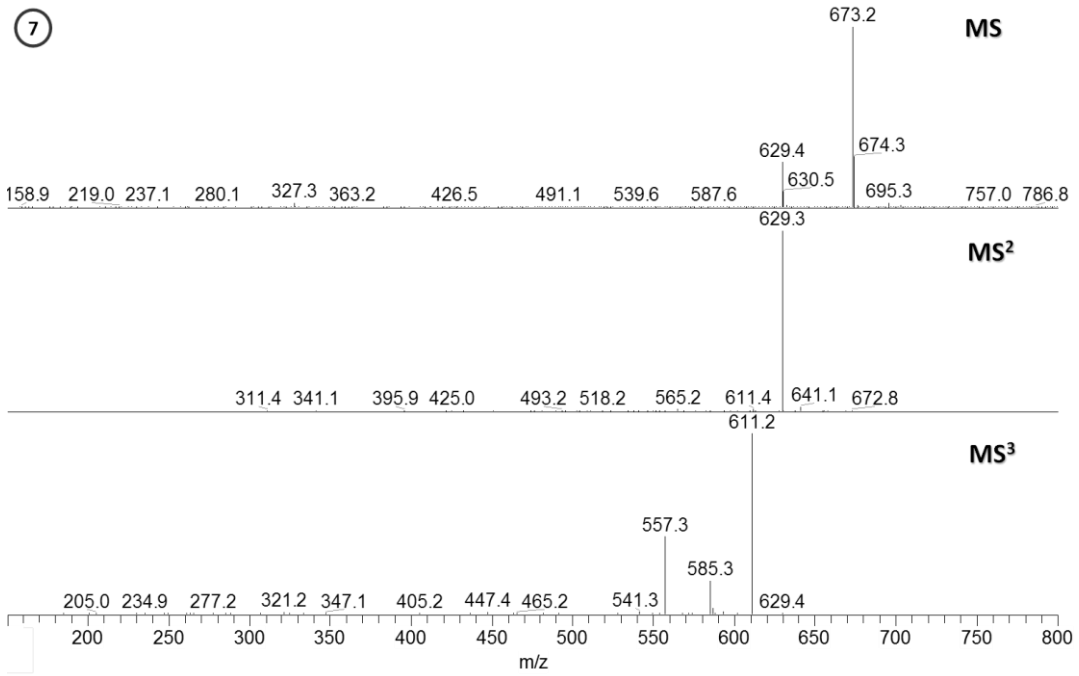
C5. Compound 5



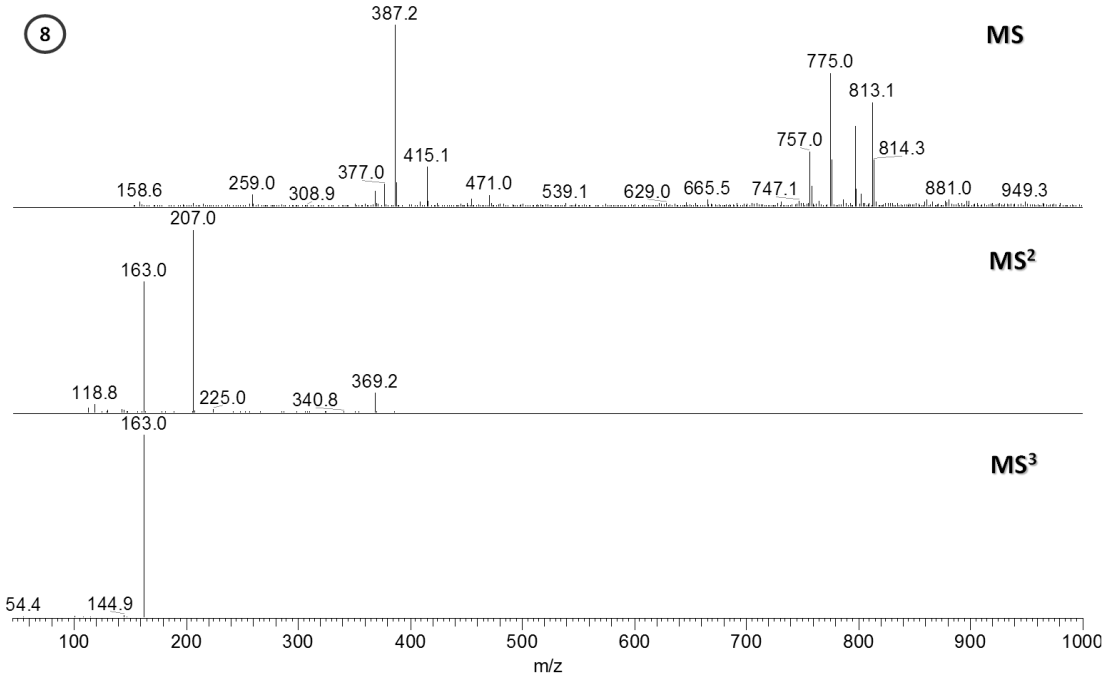
C6. Compound 6



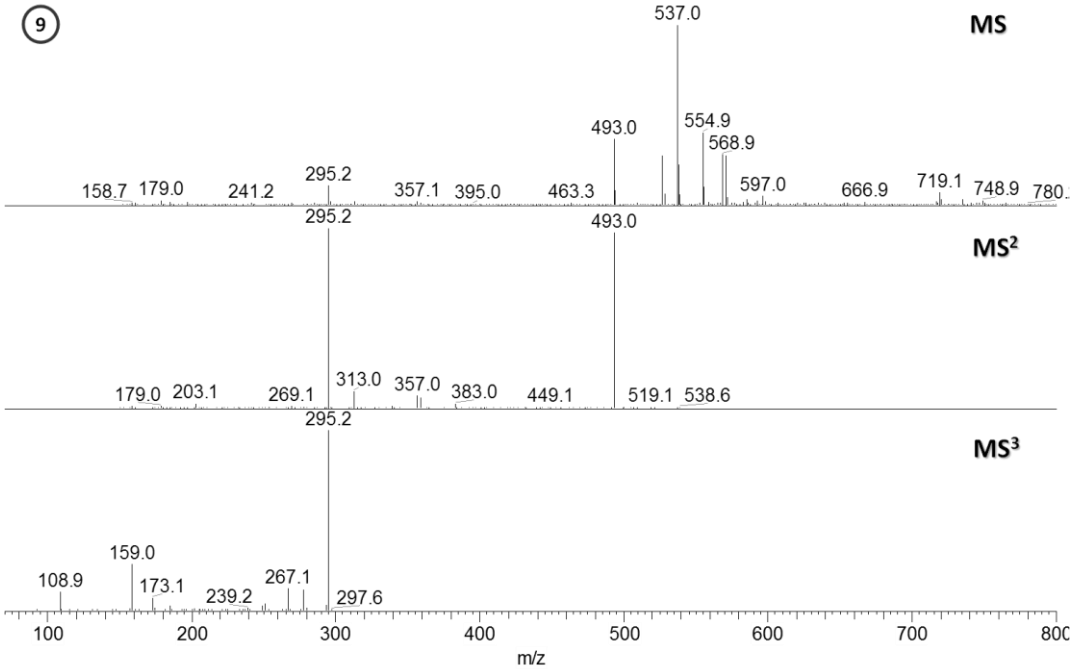
C7. Compound 7



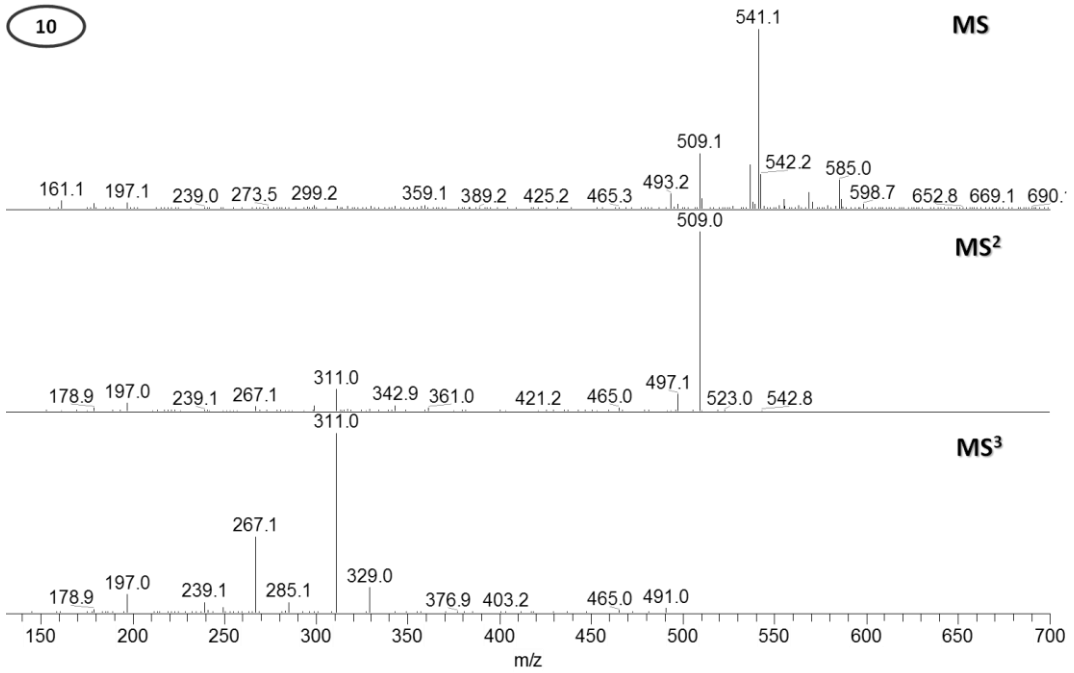
C8. Compound 8



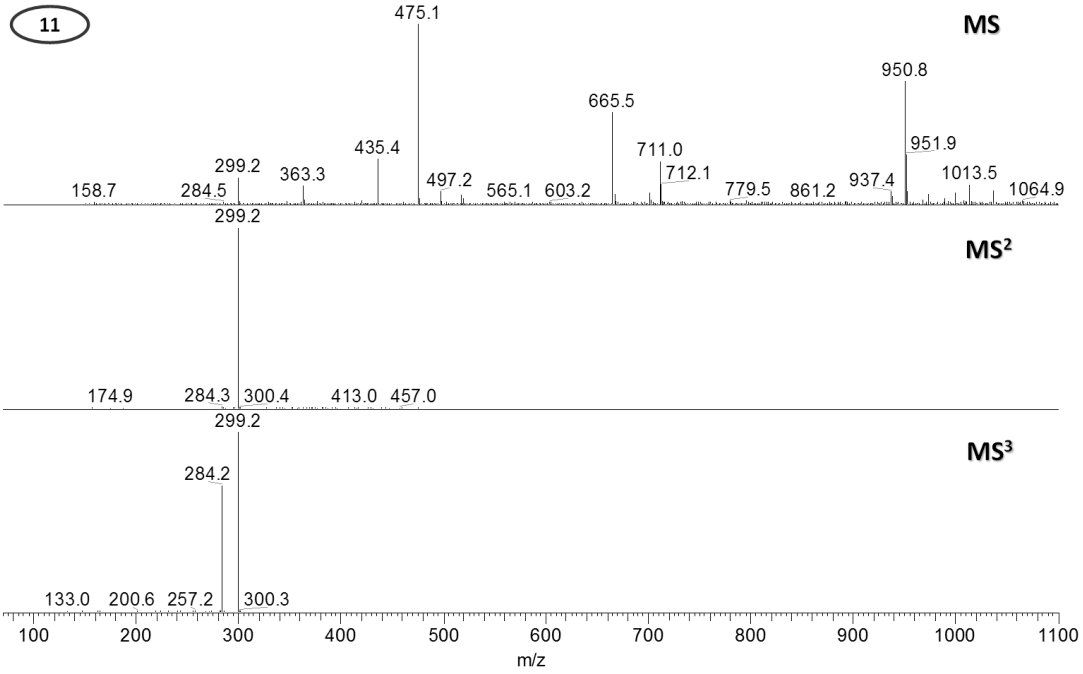
C9. Compound 9



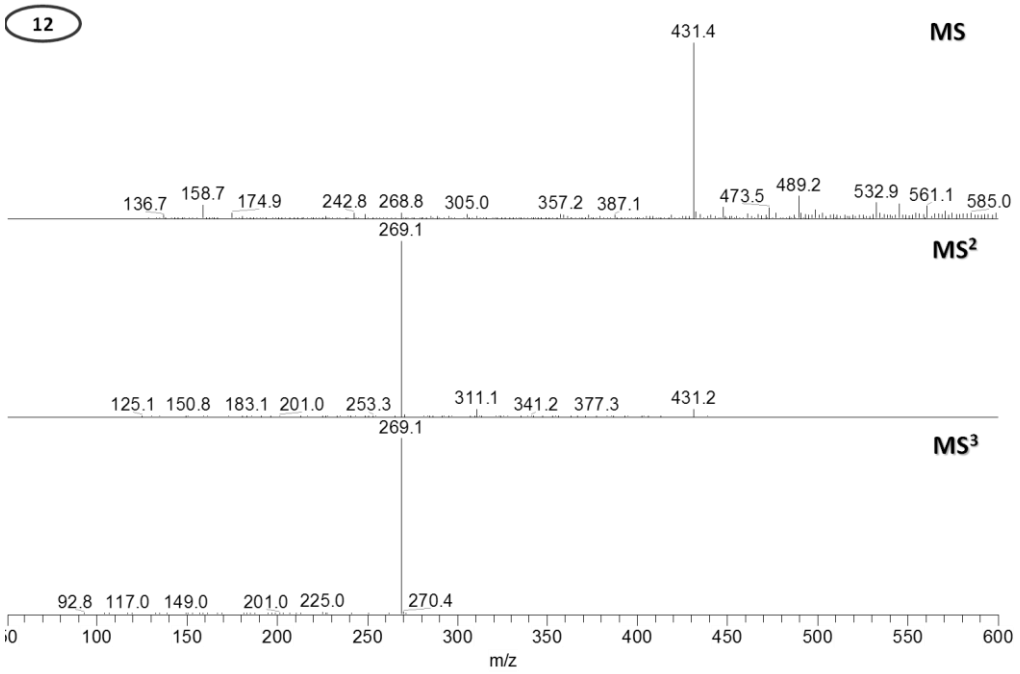
C10. Compound 10



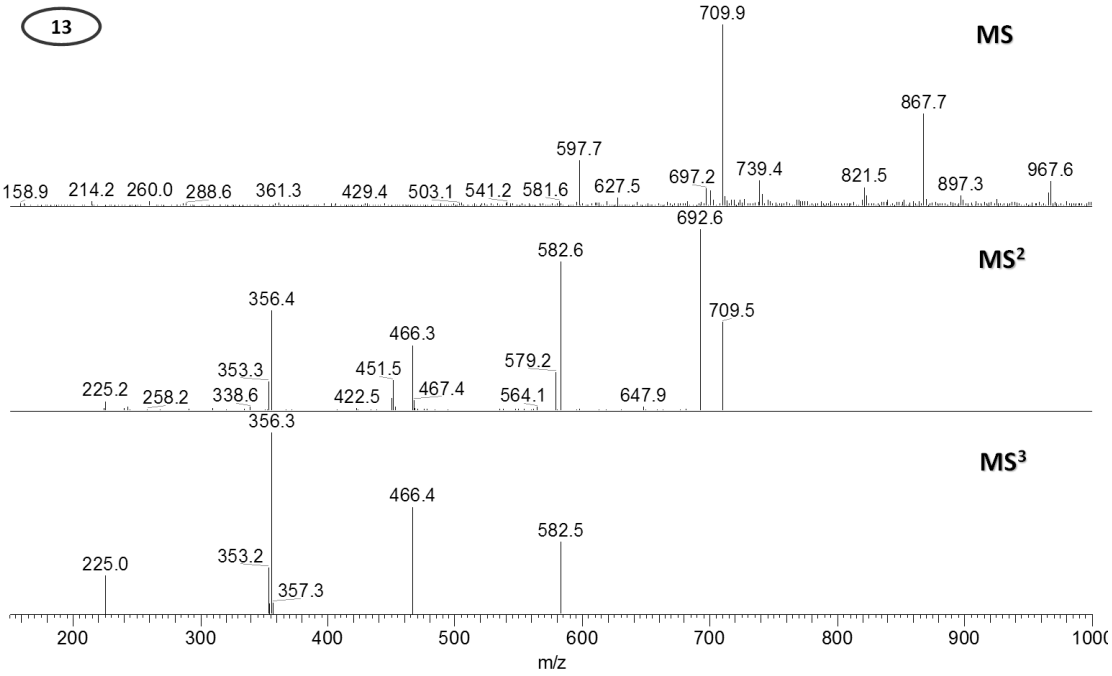
C11. Compound 11



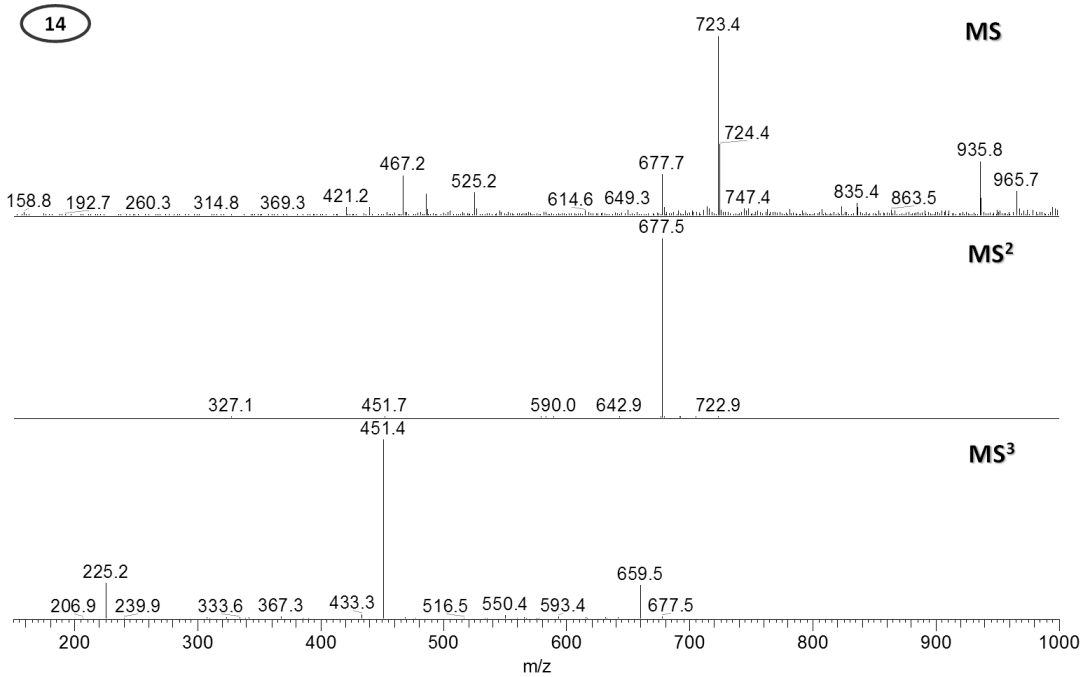
C12. Compound 12



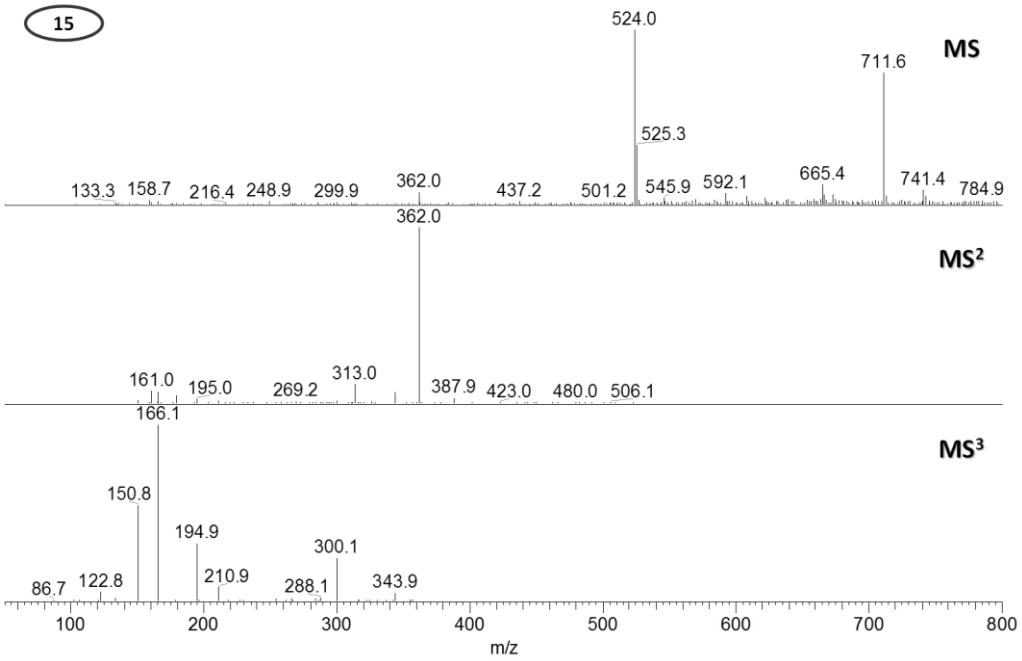
C13. Compound 13



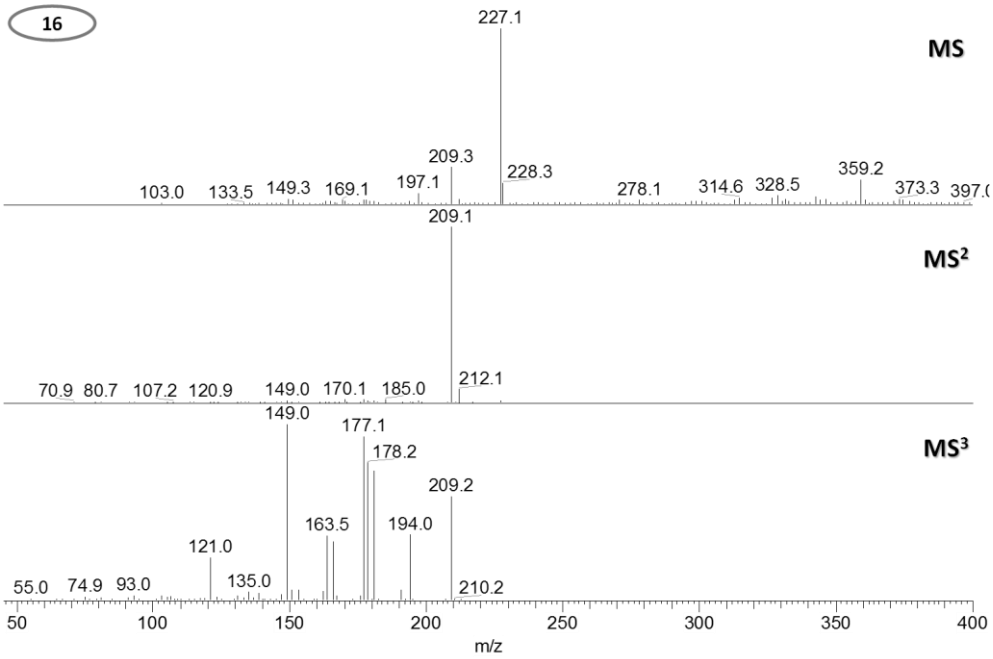
C14. Compound 14



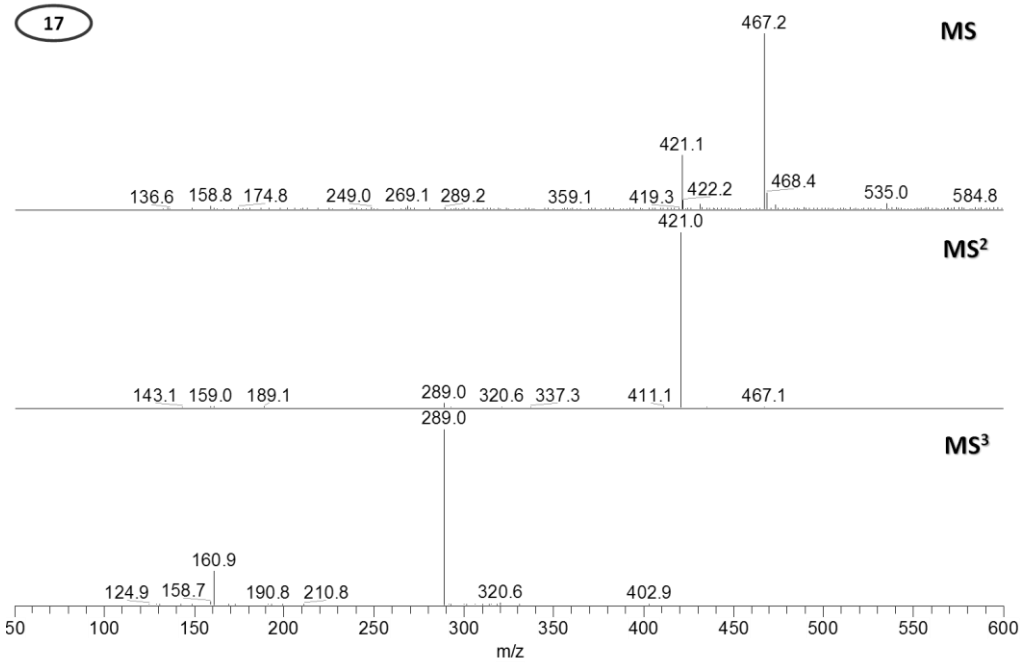
C15. Compound 15



C16. Compound 16



C17. Compound 17



C18. Compound 18